Author:
Aljaber, Maneera

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Investigation of the metabolism of ecdysteroids and their human physiological effects

Investigation of the metabolism of ecdysteroids and their human physiological effects

Maneera Yousef Aljaber

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Abstract

Phytoecdysteroids have anabolic effects in humans and especially popular among athletes as sport performance enhancers. This study investigated the metabolism of an ecdysteroid, 20-Hydroxyecdysone (20HE), and its physiological effects, at concentrations not expected to be anabolic.

The uPA (+/+)-SCID chimeric mice were administered 20HE. An excretion study was conducted on a male volunteer, after administration of low and high doses of supplement-derived 20HE. The effect of a 20HE containing supplement for two months was recorded in male athletes, with samples before and after consuming a 20HE supplement (n=4) or placebo (n=4). The detection of urinary 20HE was determined. Anthropometric changes were measured, as well as glucose, lipids and haematology, and an index of aerobic fitness (VO2 max). The hepatic transcriptome and proteome were analysed in RNA and protein extracted from the SCID mice treated with 20HE. Effects on Mitochondrial Membrane Potential (MMP) were tested *in-vitro* in human skeletal muscle cells and adipocytes treated with 20HE (5 and 10µM).

Fifteen drug metabolizing genes were overexpressed, including NOS3, and, 29 were under expressed, including CYP17A1, in the 20HE treated group. In mice, following multiple doses, 20HE was detectable for up to 24h. In a human subject, following a high dose of supplement-derived 20HE, the ecdysteroid was first detected after 2 hours, with peak detection between 4-5 hours, and remained detectable for up to 48 hours. The lower concentration was only detectable for 36 hours. 20HE supplement decreased diastolic blood pressure (p=0.05), with no significant changes in muscle mass and aerobic fitness. Seventy proteins were only detected in the 20HE group, while 37 genes were significantly differentially expressed. Upregulation of NOS3 was a consistent finding, and, validated at both the protein and RNA levels in human endothelial cells.

Low dose, supplement-derived 20HE was detectable in human urine for at least 36 hours, and its administration (2 months) was not anabolic, but, was still able to alter systemic blood pressure and the expression of vasocrine genes (NOS3).

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I want to start by giving my sincere gratitude and appreciation to my advisor Dr. Vidya Mohamed-Ali for not only allowing me to carry out a PhD but the unwavering and continuous support that she provided throughout my doctoral studies. Words do not do her justice for not only the help she offered but for her patience, motivation, and immensely vast knowledge she included as a complete package. Throughout my research and scribing this thesis, her guidance assisted and lifted me when I was at my nadir and kept me grounded when I was soaring as not to get over-confident. I couldn't imagine having a better advisor and mentor for my PhD studies and career. I need to extend my genuine appreciation to Prof. Mohammed Al-Maadheed, without his vision, support and encouragement this would not have been possible. It would be an unforgivable error on my part if I went without mentioning my supervisors: Dr David Morgan, and Dr Wael Kafineh. Their comments were both insightful encouraging. They also challenged me with their thought-provoking questions, which helped to widen my perspectives and thought processes. I am also thankful for my colleagues at the Anti-Doping Lab-Qatar for the stimulating discussions and continued support. I also acknowledge the support from the Qatar Computational Research Institute QCRI. Last but not least, I would like to thank my family: my mother, father and to my brother, sisters, husband and son for supporting and encouraging me spiritually throughout writing this thesis and my life in general.

Author's Declaration

I declare that the work in this dissertation was carried out in accordance with the requirements of the University's *Regulations and Code of Practice for Research Degree Programmes* and that it has not been submitted for any other academic award. Except where indicated by specific reference in the text, the work is the candidate's own work. Work done in collaboration with, or with the assistance of, others, is indicated as such. Any views expressed in the dissertation are those of the author.

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List of Abbreviations

20HE	20-HydroxyEcdysone
AAS	Anabolic Androgenic Steroids
ABP	Athlete Biological Passport
ADI	Athlete Diet index
ADLQ	Anti-Doping Lab Qatar
ADME	Absorption, Distribution, Metabolism and Excretion studies
ALDH	Aldehyde dehydrogenases
AR	Androgenic Receptor
ATP	Adenosine Tri Phosphate
Avg	Average
BCA	Bi-Cinchoninic Acid
BSA	Bovine Serum Albumin
CaCl2	Calcium Chloride
CAGR	Compound Annual Growth Rate
cDNA	complimentary DNA
Conc.	Concentration
CPET	CardioPulmonary Exercise Testing
cRNA	Complimentary RNA
Ct	Cycle threshold
DABG	Detection Above Background
DBP	Diastolic Blood Pressure
DMEM	Dulbecco's Modified Eagle Medium
dNTP	deoxy-Nucleotide Triphosphate
DQI	Diet Quality index
DTT	Dithiothreitol
ECG	Electrocardiogram
eNOS	endothelial Nitric Oxide Synthase (Protein)
ER	oestrogen Receptor
ER	Endoplasmic Reticulum
ERE	oestrogen Response Element
Ex/Em	Excitation/Emission
FCCP	carbonilcyanide p-triflouromethoxyphenylhydrazone
FDA	Food and Drug Administration (USA)

FFA	Free Fatty Acid
GAO	Government Accountability Office (USA)
GC	Gas Chromatography
GCMS	Gas Chromatography–Mass Spectrometry
GO	Gene Ontology
hAlb	human Albumin
HCAEC	Human Coronary Artery Endothelial Cell
HDL	high-density lipoprotein
hEGF	human Epidermal Growth Factor
HeNe laser	Helium–Neon laser
HIV	Human Immunodeficiency Virus
HKG	housekeeping gene
HSMM	Human Skeletal Muscle Myoblast
HWP	Human White Preadipocytes
IOC	International Olympic Committee
IR	Insulin Resistance
ISO/IEC	International Organization for Standardization/International Electrotechnical Commission
IVT	In-vitro Transcription
KCl	potassium chloride
KH2PO4	Dipotassium phosphate
LC	Liquid Chromatography
LCMS	Liquid Chromatography–Mass Spectrometry
LD50	median Lethal Dose
LDL	low-density lipoprotein
MAP	Mean Arterial Blood Pressure
MgSO4	Magnesium Sulphate
MMP	Mitochondrial Membrane Potential
mRNA	messenger RNA
MS	Mass Spectrometry
mt-DNA	mitochondrial DNA
MVV	Maximum Voluntary Ventilation
NADH	Nicotinamide Adenine Dinucleotide+Hydrogen
NaOH	sodium hydroxide

NCE	new chemical entities
NOS3	Nitric Oxide Synthase 3 (Gene)
NR	Nuclear Receptor
NRF	Nuclear Respiratory Factor
NSF	National Sanitation Foundation (USA)
PA	Pathway Analysis
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PD	Proteome Discoverer
QCRI	Qatar Computational Research Institute
QQQ	Triple Quadrupole
RBC	Red Blood Cells
RIN	RNA Integrity Number
RIPA	Radioimmunoprecipitation assay
RP-HPLC	Reversed Phase-High Performance Liquid Chromatography
RPM	Rotation Per Minute
RQ	Respiratory Quotient
SBP	Systolic Blood Pressure
SCID	Severe Combined Immunodeficient
SkGM	Skeletal muscle cell Growth Media
SNP	Single Nucleotide Polymorphism
SPSS	Statistical Product and Service Solutions
ss-cDNA	single stranded-complimentary DNA
TAC	Transcriptome Analysis Software
TMRE	Tetramethyl Rhodamine Methyl Ester
UHPLC/HRMS	Ultra-Performance Liquid Chromatography–High-Resolution Mass Spectrometry
uPA/SCID	urokinase Plasminogen Activator-Severe Combined Immunodeficient
VO2 max	Maximal Oxygen Uptake
WADA	World Anti-Doping Agency
xcorr	Cross correlation

Publications arising from this project:

1. Analytical strategy for the detection of ecdysterone and its metabolites in vivo in uPA(+/+)-SCID mice with humanised liver, human urine samples and estimation of prevalence of its use in anti-doping samples- submitted to Drug Testing and Analysis. 2020 (under review).

2. Dietary supplements containing low dose 20-hydroxyecdysterone induce vasodilation in recreational athletes- manuscript under preparation for American Journal of Physiology (2020).

3. Effect of 20HE on CYP17A1 and progesterone levels - manuscript under preparation - for Scientific Reports (2020)

Chapter 1

BACKGROUND AND RATIONALE

1.0 Background

1.1 Nutraceuticals. Nutraceuticals, a term first coined in the late 1980s by De Felice, combined both 'nutrition' and 'pharmaceutical', to describe the use of food as medicine (1). This practice dates back to ancient civilizations of the Nile and Indus Valley to current day. Nutraceuticals are food-derived products claiming added benefits on health, common amongst them being prevention of diseases and delaying the aging process, as well as other physiological benefits (2). Nutraceuticals are subdivided into functional foods and dietary supplements. Functional foods are basically foods that contain a component that exert at least one function on the body (3). This includes the artificial fortification of foods with vitamins or minerals during processing, most common of these being enriched flour, Vitamin D fortified milk, iodized table salt and calcium fortified orange juice. Dietary supplements is the other major class of nutraceuticals (4). While dietary supplements can be recommended by physicians, in the form of capsules or injections, they are not drugs. Unlike supplements, drugs have to be nationally/internationally certified and for this they undergo rigorous monitoring. The selection of drugs involves difficult, lengthy and capital-intensive discovery and development processes. Most times these are also inefficient since it's been reported that 95% of potential drugs fail in the development phase due to poor performance during their testing of their Absorption, Distribution, Metabolism and Excretion (ADME) (5). In addition to ADME, liberation and toxicity are also sometimes considered as part of the criteria by which New Chemical Entities (NCEs) are investigated. Criteria of NCE investigation aim to determine their kinetics and dynamics, target organ exposure, time profiles within a biological system, whether in animals or humans, to establish its efficacy, safety, potency and bioavailability, to become a potential drug (6). After undergoing in-silico pharmacokinetic studies, these compounds can be considered for multiple pre-clinical and clinical trials, after filing for regulatory status, through agencies like the Food and Drug Association (FDA). After this extensive and expensive journey, many drugs do not even make it into the market due to other limiting factors within the biological systems including SNPs and environmental factors (6). None of these tests and certification are required of supplements, as they are defined as 'over-the-counter drugs'.

Dietary supplements are considered additives to one's diet, intended to compensate for essential micronutrients that may be missing or consumed in insufficient amounts (4). The FDA legally defines dietary supplements as orally ingested, dietary ingredients that include vitamins, minerals, amino acids and herbs/botanicals, in addition to other substances taken for the purpose of supplementation of diet (7). These supplements are available in many forms making them appealing to a large proportion of the population, and include tablets, capsules, powders, energy bars, liquids and candy-like gummies (8). Terms like 'organic' and 'natural/all natural' products and increased public interest in improving and maintaining health have contributed to the reported increase in the consumption of these products, with some 49% of adults taking

dietary supplements, as per data between 2007 to 2010 (8). The main reasons for this consumption were cited as being to improve overall health and maintaining health, in percentages of 45% and 33% respectively. Consumer awareness of preventative healthcare, with a rising geriatric population and growing prevalence of chronic diseases, along with the busier daily schedule of modern life, have promoted the wish for efficient, updated approaches to nutrition (4), which have resulted in additional growth in the variety and sale of dietary supplements. It is currently estimated that the global dietary supplement market is almost $133 billion and continues to grow at a compound annual growth rate (CAGR) of 8.8%, estimated to reach more than $278 billion by 2024, according to the financial information platform marketwatch.com (9). Conversely, this may be hindered by negative publicity related to fake products. As these dietary supplements are not FDA regulated in most countries, but, rely on the assurance of manufacturers for their products' safety and effectiveness, this is basically an 'honour system' (10). Negative publicity included cases such as the death of a healthy student in the USA due to acute cardiac arrhythmia from an unintentional overdose of caffeine supplement powder (10). This is an example of a 'natural' and 'organic' everyday ingredient that actually caused serious negative effects at very large doses. While clinical trials and validated analytical methods are ideal to establish efficacy, these are not requirements for dietary supplements and yet remain advocated and recommended. In the USA, unlike for prescription drugs, dietary supplements, even those recommended by physicians to patients to compensate for any deficiency, are only presumed safe, unless reported otherwise by end-users or physicians to the FDA (10). Only after such reported adverse effect(s) is a case for this specific dietary supplement or its ingredient put under series of processes, from mitigation plans to warning and announcing to the public about these safety concerns and finally removal from the market (10). While the FDA requires dietary supplements' manufacturers to report any advert effects of their supplements, it appears that less than 1% of these serious effects get reported to the FDA (10).

1.2 Testing of Supplements. According to a metanalysis, based on 150 prevalence studies of dietary supplements among athletes, it was reported that elite athletes consume more dietary supplements than recreational athletes (11). This gave rise to the issue of ensuring the safety of dietary supplements, with third party testing of supplements being initiated. The National Sanitation Foundation (NSF), founded in the USA in 1944, was set up to ensure consumer safety. It is currently branded as NSF International and tests dietary supplements according to three reviewing criteria: label claim, toxicology and contaminants (12). In the UK the LGC Group provides a similar function. LGC was set up as the Laboratory of the Board of Excise in 1842 to test for adulteration in tobacco and regulate its production. The company still acts as the Government Chemist, providing expert opinion through independent chemical and bioanalytical testing. LGC Standards, a division of LGC Group, is the National Measurement Institute of the UK. Other, for profit testing services also emerged, such as consumerlab.com, which offered informational and quality certification in the USA, Canada

and China. A monitoring program was set up by the FDA on dietary supplements claiming weight loss or increased muscle growth being sold online and have led to them being taken off store shelves (10). That is because most of these products, instead of containing the ingredients declared on the label, are actually adulterated with prescription drugs that may have major adverse effects, including drug-drug interactions, causing serious pathology, including hepatic or cardiac toxicity (10).

The NSF has specific standards for certification of sports supplements to protect athletes from contaminants that might contain, but not declared on the label. This is especially important when considering compounds that are banned by athletic organisations, as well as some governments, such as anabolic androgenic steroids (AAS) and narcotics (12). AAS use was banned legally in 1990 in the USA under schedule III-controlled substance offense punishable by 5 and up to 10 years' imprisonment (13). Other countries followed, including Sweden in 1991, Norway in 2013, as well as the UK, Australia, Argentina, Brazil, Portugal and Saudi Arabia (14). Though AAS is banned in these countries, easy access is granted by the internet (14), as per the findings of an official investigation of the U.S Government Accountability Office (GAO) on the purchase of anabolic steroids without prescriptions, they were easily obtained without prescription using the internet (13). In this investigation 22 orders were placed and only 14 shipments were received, 10 were from foreign countries (Italy, China & Greece) and 4 were from within the USA and notably, none of these 4 contained AAS as marketed (13).

It was reported that the highest rates of use of AAS were in the countries of the Middle East and South America, and its use highest among recreational athletes (14). Amongst recreational athletes, bodybuilders are the most likely to use a combination of different substances including proteins, pills and amino acids (Figure-1) (14). The strains caused by the combined excessive use of these supplements on the physiology is immense and long-term use of proteins as supplements, instead of protein-rich foods, can increase risk of osteoporosis and cause the deterioration of existing kidney problems (15). In addition, treatment fatigue has also been reported, which is defined as the decreased adherence to treatment due to many reasons, including adverse effects, inconvenient scheduling and dosage (16). This is apparent in the sport of bodybuilding, often 'prescribed' a number of pills and shakes, that they expected to adhere to as part of their training. One example is presented in (Figure-1), where an advertised growth hormone and testosterone stimulating supplement's recommended dose is 3 capsules in the morning and 4 in the evening.

A study from Germany of 164 young elite athletes reported that 80% were using supplements, with significantly increased prevalence of use among older athletes, mainly for health-related reasons (17). The study also reported that only 36% of these elite athletes were familiar with the possibility of inadvertent doping due to contamination in these supplements purchased from pharmacies and supermarkets (17). Another study surveyed 77 professional swimmers in Australia and reported that 94% were consuming dietary supplements, with some concerned about the safety and risk of inadvertent doping (18).

a)

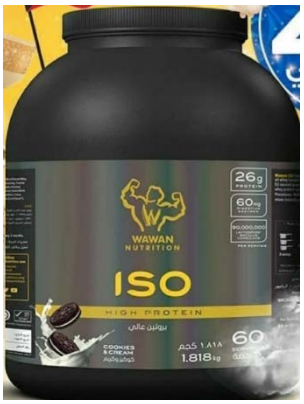
b)

c)

d)

e)

f)



g)

h)

(Figure-1) An example of a set of supplementations recommended to a body builder by a person identifying himself as a certified nutritionist\Coach. Advertised claims on the supplements include: Growth hormone and testosterone stimulant capsules (a) and antiaging, cardiac strengthening, muscle mass increasing and fat burning & converting to energy powder (h). Other supplements included branched chain amino acids, fatty acids, protein powders and vitamins (b-g).

1.2.1 World Anti-Doping Agency (WADA). WADA was established by the international Olympic Committee (IOC) in 1999 for the promotion and coordination of the fight against doping in sport. As part of its role WADA accredited several laboratories to test for the substances that are classified as dopants. Laboratories that are WADA accredited for testing of prohibited substances in sport go through an extensive and rigorous process to obtain this accreditation. This starts with expression of interest to setting up the analytical methods, application of them in a standardized manner and acquiring ISO/IEC 17025 accreditation (19). Currently there are 30 WADA accredited labs in 27 countries worldwide. In addition to the accredited laboratories, a list of prohibited substances and methods in sport is published every year by WADA that clearly states and identifies all substances that are banned and shall not be consumed by competing athletes in and out of competition. It is classified in different categories such as steroids, stimulants, manipulation or gene doping (Table-1). In addition to the prohibited list, WADA also publishes a monitoring list (Appendix 1), that is also updated annually. This list is for substances that are not prohibited, but, require monitoring for patterns of abuse by competing athletes. Such substances include caffeine, which is considered a food additive, dietary supplement and drug.

The main objectives of WADA are to ensure fair play, clean competition and protection of athletes. While some professional athletes wilfully abuse illegal substances to gain a competitive advantage, in most cases this appears to be inadvertent. Examples of this are innumerable, including that of Maria Sharapova, the female tennis player, who tested positive in 2016. The athlete had been taking meldonium for 10 years without falling foul of WADA. However, meldonium was added to the WADA 2016 prohibited list, and in that year the athlete tested positive for it, because she failed to read the updated list (19).

In 2009 WADA approved the profiling of haematological, biological variables, or biomarkers, of competing athletes and the Athlete Biological Passport (ABP) was developed to monitor these blood biomarkers, and comparing it to a population. First, a baseline was created for each athlete over time, from at least 4 blood samples, after which any variation could indicate the effect of doping (21). ABP provides a new approach in the fight against doping since it is investigating the effects exerted by a prohibited substance, rather than investigating the presence or absence of the prohibited substance or its metabolites in the blood or urine.

Recently the IOC released a statement emphasising the need for sport-specific research on supplements that uses appropriate research experimental models and methods, taking into account the real-life practices of athletes (22). A first step in the recommendation was to conduct a detailed comprehensive nutritional assessment of the athlete, that included dietary evaluation, clinical examination and medical history, taking into consideration age, gender and ethnicity (22). This can be viewed as an application of Diet Quality Indices,

which are algorithms that aid in the quantification of adequacy between nutrient intake and reference intakes that assures optimal health (23). A recent report suggested athlete-specific diet index ADI as an efficient tool for the assessment of athlete's quality of diet and to provide guided support of nutrition intervention (24). All athletes recruited in the study reported ADI as relevant and easy, which is a good marker for compliance and motivation of use.

Class	Category	Examples	Class	Category	Examples
S0	Non-approved substances	Discontinued drugs; designer drugs	M1	Manipulation of blood and Blood components	Autologous or heterologous Blood transfusion
S1	Anabolic agents	Epitestosterone; methasteronep oxabolone	M2	Chemical and physical Manipulation	Sample substitution and/or adulteration
S2	Peptide hormones, growth factors, Related substances, and mimetics	Erythropoietins (EPO); Hypoxia-inducible factor (HIF) activating agents	M3	Gene and cell doping	Use of nucleic acid that alter genetic code or sequence
S3	Beta-2 agonists	Formoterol; Indacaterol	P1	BETA-BLOCKERS	This class is specific to few sports
S4	Hormone and metabolic Modulators	Aromatase inhibitors; Selective oestrogen receptor modulators (SERMS)	S5	Diuretics and masking agents	Acetazolamide; amiloride
S6	Stimulants	Amfetamine; Benzfetamine;	S7	Narcotics	Buprenorphine; Diamorphine (heroin)
S8	Cannabinoids	Cannabidiol.	S9	Glucocorticoids	Cortisone; Deflazacort

(Table-1) Classes and categories detailed in the WADA prohibited list of the year 2020 (appendix1)

1.3 Sport performance enhancers. Mediators of sporting performance and exercise have become the focus of much research, both, from the competitive and health points of view. Swiftly following on from this has been the search for compounds that could act as exercise mimetics and enhancers of sporting performance, often effecting changes in body composition and energy metabolism, at least in part, mediated by alterations in mitochondrial and vascular functions (11). The most prevalent families of compounds that fall under these criteria are the anabolic androgenic steroids (AAS), such as testosterone analogues, and stimulants, such as caffeine and amphetamines (7). These are often components of many marketed dietary supplements (9). However, as both AAS and many stimulants are either prescription only drugs or prohibited substances in sporting competition, legal and easily obtainable substitutes for these are vigorously sought (25).

Amphetamines are perhaps the most widely abused drug in baseball in USA and are especially popular amongst young athletes (26). Erythropoietin is another substance with a proven track record of abuse, highly effective as an aerobic enhancer and has been linked to many deaths of athletes in cycling and other endurance sports (27,28). Caffeine is a widely consumed, socially acceptable beverage, forming part of the diet of a large proportion of the adult population. There is clear evidence showing caffeine to be an ergogenic aid in several types of sport (29). Even relatively moderate intakes of caffeine (≈3 mg/kg body mass) can improve performance of a range of sporting disciplines including endurance sport, stop-and-go events and high intensity sport (30-33). It is freely available from online outlets in formulations promoted as a natural enhancer of performance during exercise, mostly for ergogenic effects among adolescents (10). It was reported that some of these caffeine formulations contain up to 30 times the lethal dose of caffeine (10).

AAS are one of the most popular doping substances. They are synthetic derivatives of the male hormone testosterone. They have significant effects on human physiology that are beneficial for athletic performance (34). Their use, both as sport performance enhancers and for improving body shape, by increasing muscle, was first reported in the 1950s and rapidly spread amongst athletes, despite being denounced by physicians (35). It has been shown that short-term administration of AAS to athletes increased strength and bodyweight (36). Strength gains of up to 20% of baseline strength and up to 5 kg bodyweight, that may be attributed to an increase of the lean body mass, have been reported (37). A reduction of fat mass has been shown by some studies but this is not a consistent finding (38). AAS affects erythropoiesis, through the stimulation of erythropoietin synthesis and red cell production and blood haemoglobin levels, but whether this improves endurance performance is still unclear (39). The effects of AAS on metabolic responses during exercise training and recovery are also debatable.

AAS act principally via the androgen receptors, with involvement of some other mechanisms of action, dependent on variations in the steroid molecule and receptor affinity (40). Other mechanisms include the effects of the enzymes 5-alpha-reductase and aromatase (41). 5-alpha-reductase has an important role in converting AAS into dihydrotestosterone (androstanolone), which then acts in the cell nucleus of target organs. Aromatase converts AAS to oestradiol and oestrone, antagonistic action to oestrogens and a competitive antagonism to the glucocorticoid receptors. AAS-induced increment of muscle tissue is attributed to hypertrophy and the formation of new muscle fibres, in which key roles are played by the androgen receptors (42).

The main adverse effects of acute and chronic AAS abuse are increased sexual drive, acne vulgaris, hirsutism and propensity for aggressive behaviour (43). AAS administration inhibits endogenous production of testosterone and gonadotrophins, an effect that persists for months after drug withdrawal. Worsening of cardiovascular risk factors following AAS, such as elevated blood pressure, increased triglycerides and lowered HDL-cholesterol have been reported (44). In male athletes AAS did not seem to affect cardiac structure and function, though in murine studies these drugs have deleterious effects on heart structure and function (45). The effects of AAS on the cardiovascular system are proposed to be mediated by the occurrence of atherosclerosis, perhaps due to the dyslipidaemia, thrombosis, vasospasm or direct injury to vessel walls, or a combination of these (46). Psychological effects, leading to abnormal and anti-social behaviour, are strongly associated with AAS abuse, apparently increasing aggression and hostility. Depression also seem to be related to dose and drug dependence, though only reported in a small number of AAS users (47). Many other adverse effects have been associated with AAS misuse, including disturbance of endocrine and immune function, alterations of sebaceous system and skin, changes of haemostatic system and urogenital tract. It is also possible that these adverse effects are an underestimate because of the relatively low doses administered in most studies, as for ethical reasons studies do not approximate doses used by illicit steroid users (48).

Apart from the endogenous AAS, these steroids can be synthesized, in order to evade doping control tests. This was first discovered in 2003 with the discovery of the designer steroid tetrahydrogestrinone (49,50). Despite much effort and research the detection of these ever increasing list of designer steroids this still remains one of the main analytical challenges in the doping control field. Other potential performance enhancing substances are constantly being proposed. A recent class of substances that are being revisited are the ecdysteroids.

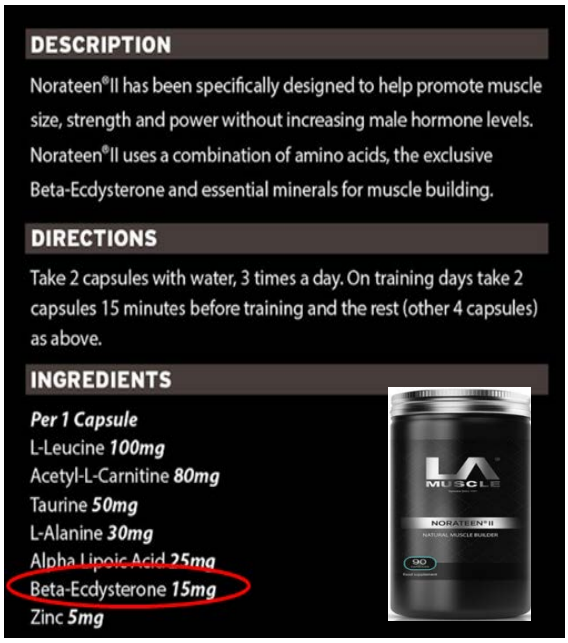
1.4 Ecdysteroids. Plant steroids were classified into 7 groups according to Kreis and Müller-Uri (2010) depending on their function or chemical structures (51). These plant steroids groups include: Phytosterols; campesterol and stigmasterol, Brassinosteroids; Brassinolide, castasterone and Phytoecdysteroids; 20-Hydroxyecdysone (20HE), which is the substance of interest in this study (51). Phytoecdysteroids, and mainly 20HE, have had a revived interest in their uses as potential aids to weight loss and sport performance enhancement especially among recreational and elite athletes. Ecdysteroids are polyhydroxylated ketosteroids that were initially isolated from arthropods, with a role in the moulting process (52) Phytoecdysteroids, have subsequently shown to be more abundant in plants, with, as yet, unidentified hormonal functions (53). However, this family of phytosteroids have gained interest because of their purported anabolic effects in mammals. They are especially popular among recreational and elite athletes who consume these, either in their pure forms or formulated as a component of dietary supplements, as sport performance enhancers (54). Many of these ecdysteroids are readily available from numerous online outlets (Figure-2).

a)

b)

c)

d)

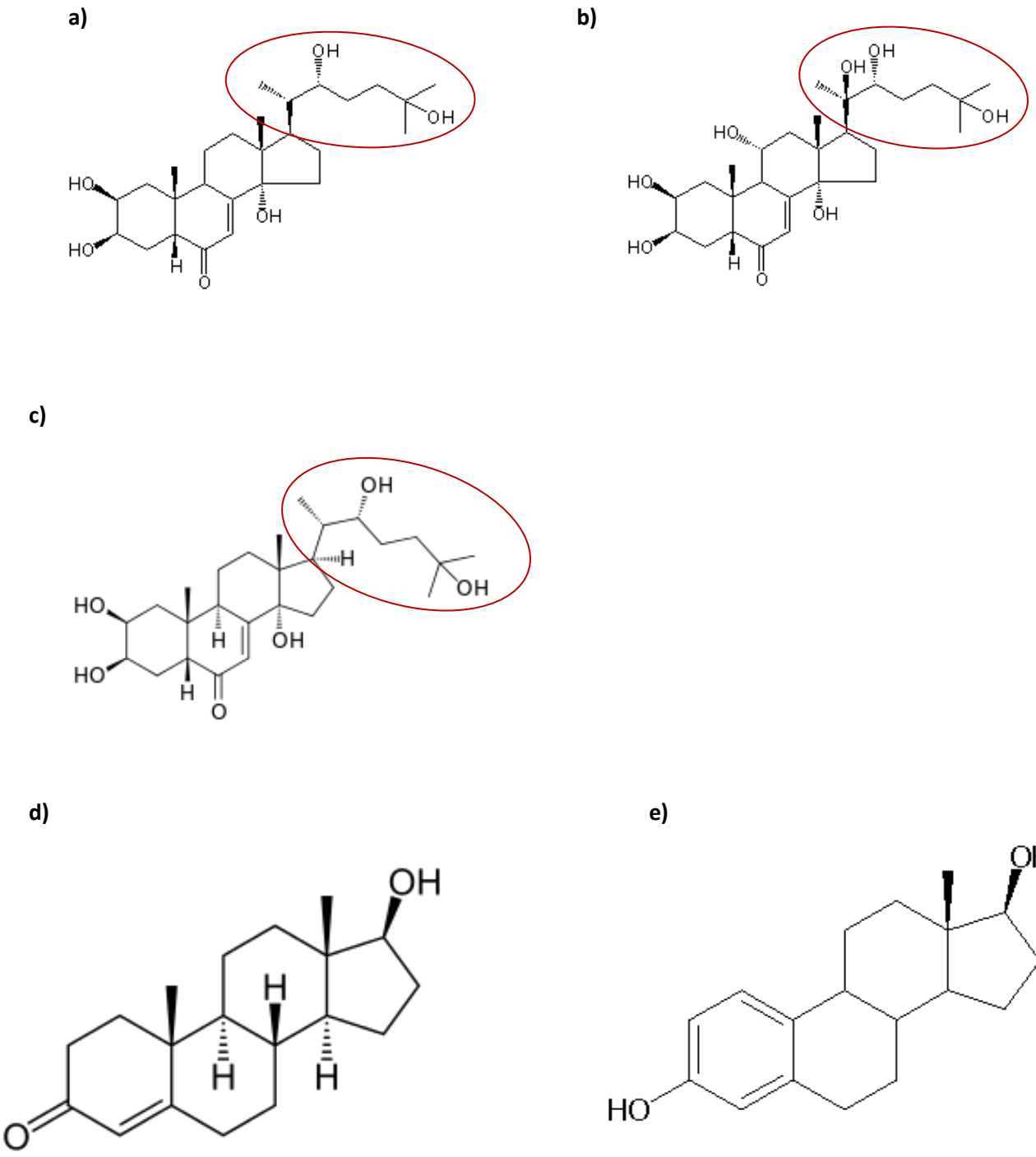


(Figure-2) Supplements claiming to contain ecdysteroids available online. Animal Stack (a), Natural sterol complex(b), Desire-X (c) and Norateen II (d) (55).

1.4.1. Origin, biosynthesis and diversity amongst species. Ecdysteroids are polyhydroxylated ketosteroids that were initially isolated in insects where they regulate moulting and reproduction (52). Closely related molecules to ecdysteroids have been found in plants, called phytoecdysteroids, where their role is as yet unclear (53).

However, their (phytoecdysteroids) possible use as pesticides has been of some interest, as they have the ability to interfere with insect development and reproduction and cause premature metamorphosis (56). But the chemical complexity of ecdysteroids and the environmental and metabolic burden of insects has made this research slow. A live database of all reported ecdysteroids is maintained by the University of Exeter (ecdybase.org), which currently lists more than 500 of them (57).

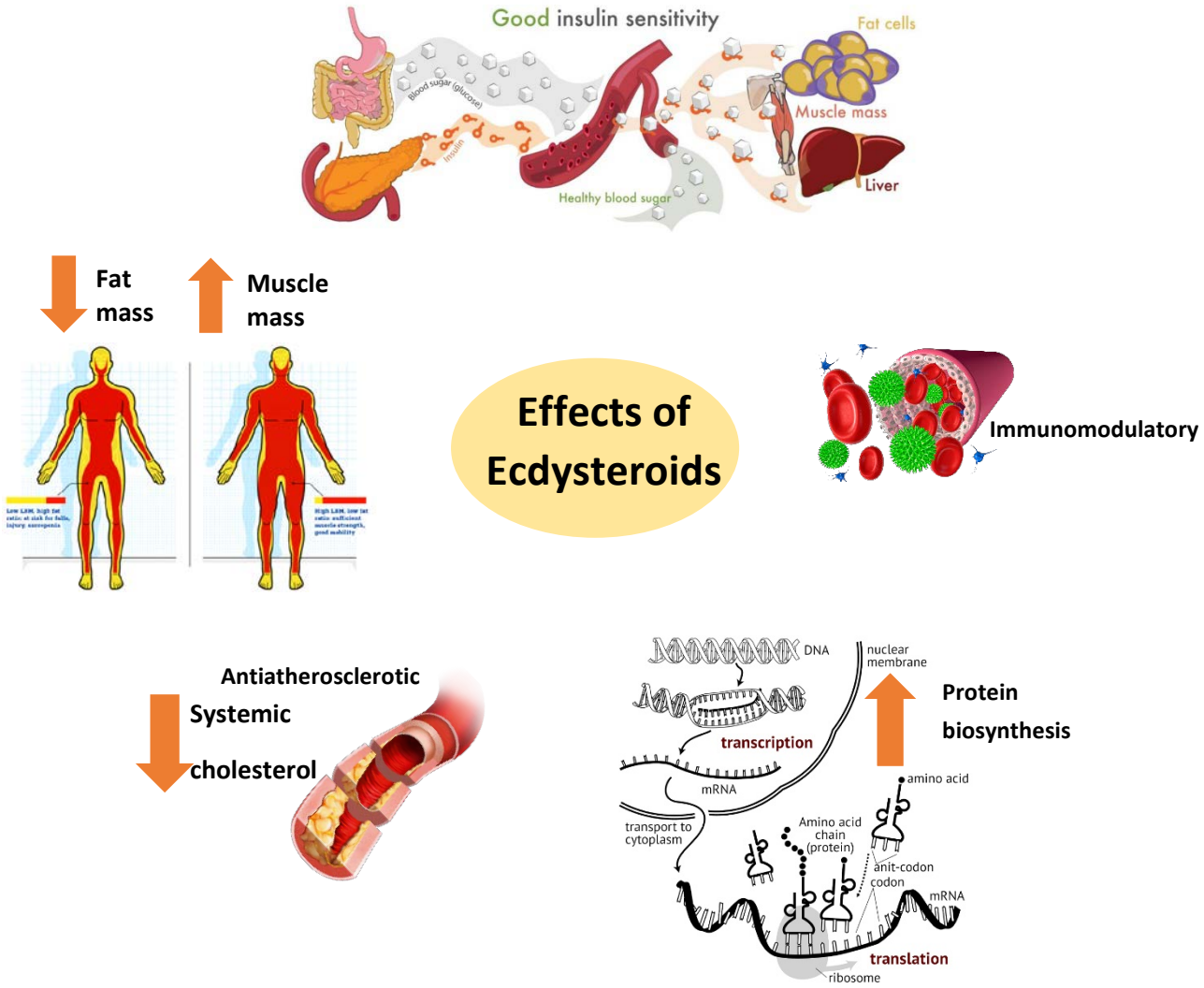
Chemically, phytoecdysteroids comprise a family of compounds called triterpenoids, which includes saponins, phytosterols and the phytoecdysteroids (58). Plants are able to completely synthesize ecdysteroids from mevalonic acid and cholesterol (59). Further hydroxylations at positions C2, C20, C22, and C25 produce the most reported of the species, 20 hydroxyecdysone (20HE;60). The site of biosynthesis of phytoecdysteroids within the plant appears quite wide spread. Only a minority, about 6%, of all plant species produce phytoecdysteroids (61). However, some species, such as spinach and quinoa have significantly elevated levels of phytoecdysteroids (62). Despite the name, ecdysteroids, insects are not able to carry out de novo synthesis of these steroids but need to consume phytosterols and then convert these to ecdysteroids (60). The sterol structure is modified to produce ecdysteroids. The major difference in the structure between these and the mammalian steroid hormones is the polyhydroxylated side chain (Figure 3;60).



(Figure-3) Chemical structure of ecdysteroids and mammalian steroid hormones. The top panels show some of the common ecdysteroids with the polyhydroxylated side chain circled in red a) 20 Hydroxyecdysteroid b) Turkesterone and c) Ecdysone. The bottom panels show the mammalian steroid hormones, testosterone d) and 17-β oestradiol e) (60).

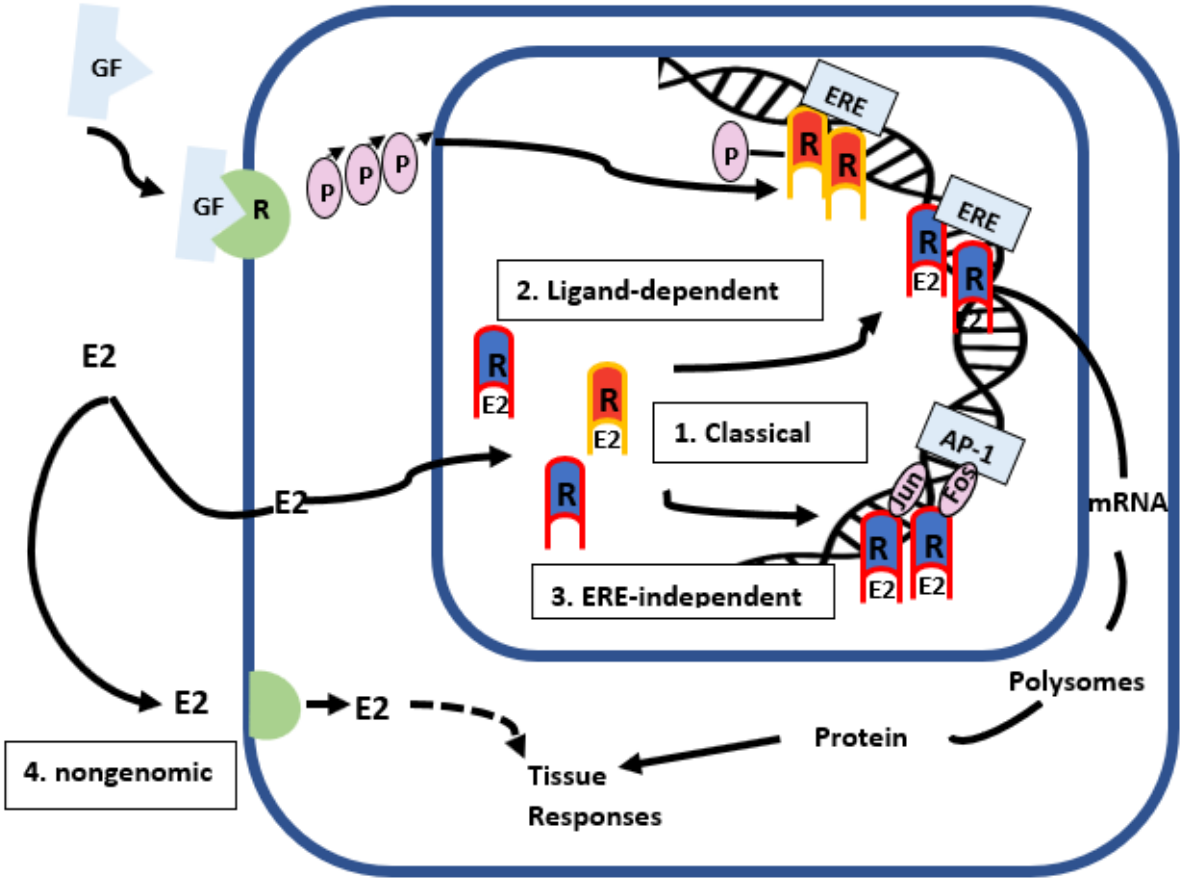
1.4.2 Effect on sport performance and health. It has been reported that dietary supplement consumption among professional and recreational athletes is between 40 to 88% (63), with an increasing number of them containing ecdysteroids. This is because ecdysteroids are widely marketed as natural anabolic agents based on some data from both murine and human studies. Their anabolic effect is reported to be comparable to, and possibly even greater than, many AAS. Studies on rodent models revealed elevated muscle fibre size in groups treated with ecdysteroids, in comparison to placebo, through increased protein biosynthesis. However, their ability to elevate protein biosynthesis does not appear to be targeted and has also been noted to lead to hypertrophy of other organs, such as heart and liver (54). The first of such reports showed enlargement of the liver in mice. This raises the question of their safety and possible adverse effects on health. Reports indicate that Turkesterone is the most potent anabolic ecdysteroid, however, 20-hydroxyecdysone is the most studied, possibly due to its higher availability synthetically in supplements (56), and also in many 'super' foods, such as quinoa. Quinoa (Chenopodium quinoa) is a pseudo-cereal which was consumed by Native Americans for a long time but has recently been rediscovered by the food industry due to its protein quality, as well as a source of minerals, vitamins and flavonoids (64). Quinoa contains high levels of phytoecdysteroids and there is some evidence of it having anabolic effects. In addition to the anabolic effects, some studies have reported that 20-hydroxyecdysone plays a role in the control of glucose homeostasis and more possibly in the prevention of diet-induced obesity in mice (Figure 4; 64). In rats it has been shown to lower experimentally induced hyperglycaemia, and in mice it enhances insulin stimulated hepatic glucose incorporation into glycogen, as well as glucose utilization by many other tissues. Most of these effects appear to be via increased insulin sensitivity (51). Further health claims include reduction in hepatic cholesterol biosynthesis and increased catabolism, promoting conversion to bile acids and thus reducing systemic cholesterol. This effect on cholesterol is thought to be the basis for their anti-atherosclerotic effects (51). Other health benefits include the promotion of wound healing by stimulating keratinocyte differentiation. Numerous studies also show some evidence for the ecdysteroids being immunomodulatory in rodents and humans (51).

1.4.3. Toxicity and Adverse effects. The ecdysteroids tested so far appear non-toxic, with, LD50 of 6.4 g/Kg after intraperitoneal injection and 9 g/Kg after oral application of 20-hydroxyecdysone to mice (55). 20-hydroxyecdysone was not embryotoxic when injected into developing chicken eggs. While available data from reports indicate that 20% of hepatotoxicity cases is caused by herbal and dietary supplement-induced liver injury these have not, to date, specifically been attributed to ecdysteroids (65).



(Figure-4) Reported effects of ecdysteroids in mammals. Ecdysteroids gained interest due to their anabolic effects (54,66,76) which is triggered by protein biosynthesis (54). They play a role in the control of glucose homeostasis where it was reported to reduce experimentally induced hyperglycaemia and enhancement of the incorporation of glucose into glycogen in the liver that stimulated by insulin (64). This effect correlates to reported increased insulin sensitivity (26). Reduction of systemic cholesterol caused by the reduction of its hepatic biosynthesis which form the basis of its anti-atherosclerotic effects (51). Several studies report immunomodulatory effects in rodents and mice and other effects include promoting wound healing triggered by the stimulation of keratinocyte differentiation (51) and prevention of diet-induced obesity in mice (64).

1.4.4. Ecdysteroids receptors. Phytoecdysteroids have recently been shown to function in humans through the oestrogen receptor (66). Parr et al reported data from *in-silico* docking experiments that ecdysteroids, unlike AASs, bind the oestrogen receptor (ER), especially ERß, rather than androgenic receptor (AR) (66). The consequences of this are yet to explored, as the ER is widely expressed in tissues and its signalling is complex (Figure-5). There are several distinct pathways by which oestrogens and ERs could have cell-specific effects on a variety of physiological endpoints including regulation of mitochondrial biogenesis and activity. Oestrogens regulate gene transcription by the classical genomic mechanism of binding to oestrogen receptors α and β (ERα and ERβ) as well as by non-genomic pathways, via plasma membrane-associated ERs that activate intracellular protein kinase-mediated phosphorylation signalling cascades. The most likely signalling pathways for ecdysteroids acting via the ER may be in the ligand independent manner, or the non-genomic pathway. However, this is yet to be investigated. As oestrogens, in addition to their role as sex steroids, are important regulators of metabolism and impact on diseases such as obesity and insulin resistance (IR), these may also be affected by ecdysteroids binding to the ER. While ERα seems to have a protective role in such diseases, the function of ERβ is not clear. There is some data that ERβ-deficiency may protect against diet-induced IR and glucose intolerance, via a pathway that involves PPARγ signaling in adipose tissue (67). The identification of ERα and ERβ within mitochondria of various cells and tissues may especially impact on the regulation of the transcription factors such as Nuclear Respiratory Factor-1 (NRF-1, *NRF1*). NRF-1 promotes transcription of mitochondrial transcription factor Tfam (mtDNA maintenance factor, also called mtTFA) and then Tfam targets mtDNA-encoded genes. The nuclear effects of oestrogens on gene expression directly controlling mitochondrial biogenesis, oxygen consumption, mtDNA transcription, and apoptosis are yet to be studied. Overall, the many direct and indirect effects of oestrogens on mitochondrial activities is still largely unexplored. The effects of its activation of ER by ecdysteroids is even less investigated, especially in the cells of insulin responsive tissues, such as adipose tissue and skeletal muscle.



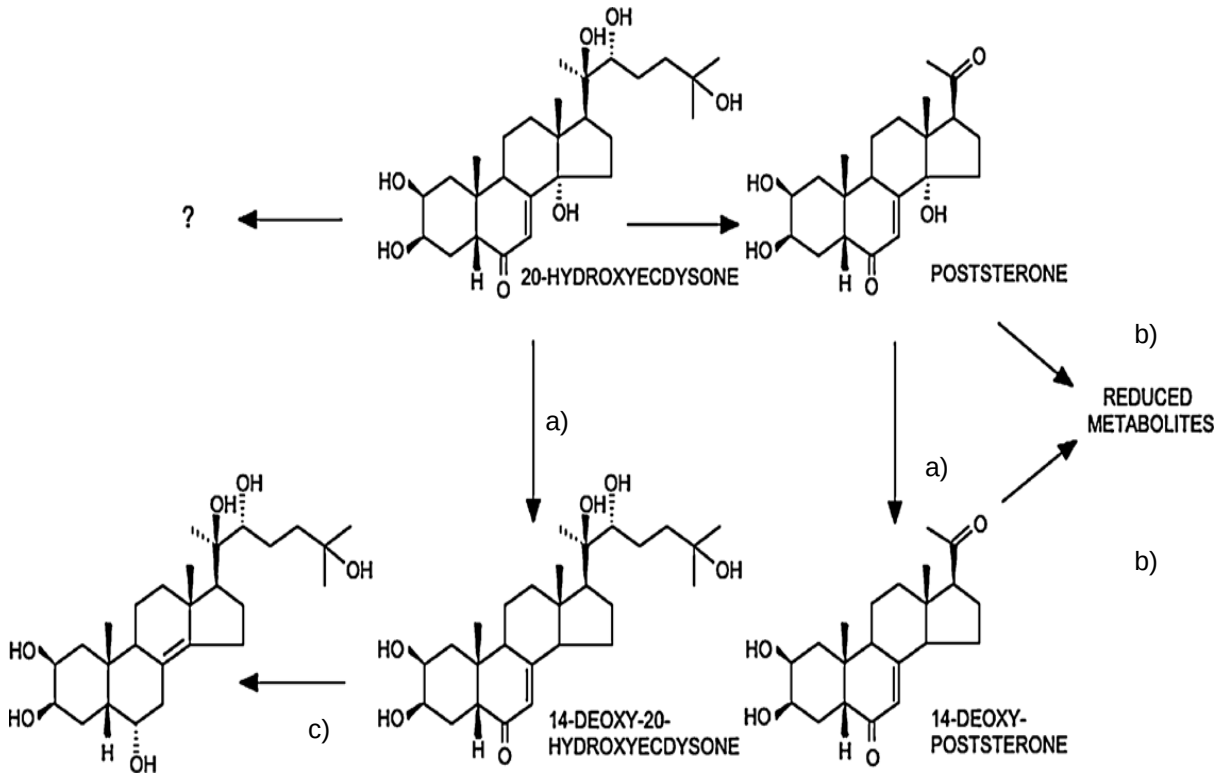
(Figure-5) Oestrogen Receptor (ER) signalling. Four mechanisms through which oestrogen signals includes: 1. Classical ligand-dependent. This is common to all Class 1 nuclear receptors (NRs). In the absence of ligand, the receptor is appropriated into an inhibitory complex and is stored within nuclei of target cells. Ligand binding leads to an up- or down-regulation of gene transcription and followed by tissue responses. This process causes changes in the conformation of the ER, stimulates homodimerization, binding to specific DNA response elements (ERE) in cis-acting enhancers within regulatory region of genes and gene transcription. 2.Ligand independent. Even if E2 is not present some GFs, such as IGF-1 are able modify the receptor function and that of secondary signalling molecules such as cAMP, that go on to activate various kinases pathways, leading to phosphorylation (P) and activation of ER at ERE-containing promoters in a ligand-independent manner.3. ERE-independent. In those genes that lack ERE-like sequence in their promoters, E2-ER complex alters transcription of genes containing alternative response elements, such as AP-1, through association with other DNA-bound transcription factors, such as Fos or Jun, that tether the activated ER to DNA, resulting in up-regulation of gene expression. 4. Non-genomic. In order to elicit rapid effects of E2, for example on blood vessels, the ligand activates a recognised membrane-associated binding site to generate rapid tissue responses, such as the activation of eNOS by MAPK/PI3K/Akt pathways (68).

***1.4.5. Metabolism of ecdysteroids*.** Earlier studies in mice injected with significant amounts (1 mg/animal) of ecdysone or 20HE (55), with faecal and urinary samples analysed by RP-HPLC over 48 hr, showed the parent steroid excreted unchanged, as well as about 10 metabolites, with the major ones having the unsaturated ketone on ring B reduced. This was unlike the reduction reactions on ring A shown during the catabolism of vertebrate cortisol or androgens. A more recent report looking at the murine metabolism of 20-hydroxyecdysone injected intraperitoneally (in faecal and urinary samples) showed that the primary reactions produce three major metabolite groups (Figure-6). Table-2 summarises all the metabolites reported in the various mammalian species reported to date and their stability, if known.

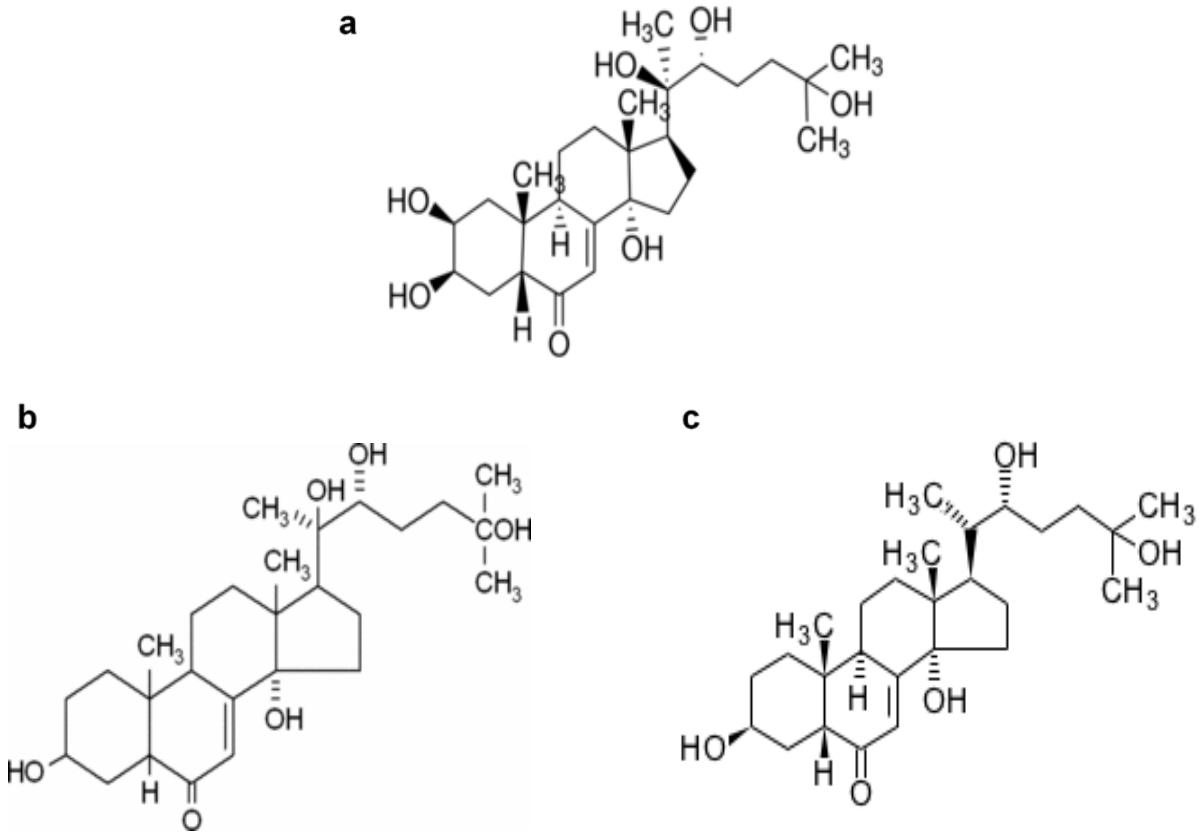
The identified metabolites to date in human subjects are 2-deoxy-20 hydroxyecdysone, 2-deoxyecdysone (Figure-7) and 14-deoxy 20hydroxyecdysone (69,70). The structures of the former two have been reported. Both these studies have used only one volunteer each being administered one dose, 20 mg. Whether there are age, gender and ethnicity associated differences in the metabolism of ecdysteroids in humans are not known currently. Furthermore, the metabolic fate of other ecdysteroid species, such as Turkesterone, are also yet to be investigated. Thus, there is a need for further studies to address these issues urgently in the face of the increasing popularity of these phytosteroids.

Species	Ecdysteroid	Parent steroid detection	Metabolites (urinary)	Reference
Calves	20HE	Up to 24h	14-deoxy-20-hydroxyecdysone (6 days) 26-dihydroxyecdysone 14-d 20 26-dihydroxy	Destrez et al. (71)
Mice	20HE	Yes	Several metabolites from 1. 14-dehydroxylation 2. Complex reduction of the 7-en-6-one chromophore 3. Epimerization	Girault et al. Kumpun et al. (72,73) Lafont et al. (55)
Rat	20HE	Yes	3 metabolites from the loss of 6-keto group followed by reduction of 7-ene bond	Ramazanov et al. cited in Lafont et al. (55)
Human	20HE	Yes	2-deoxy-20 hydroxyecdysone 2-deoxyecdysone	Tsitsimpikou et al. (69)
	20HE	Yes	14-deoxy 20hydroxyecdysone	Brandt et al. (70)

(Table-2) Metabolites of Ecdysteroids in mammals. Reports from claves study showed 3 metabolites with one metabolite stable for 6 days (71). Three metabolites were reported in mice (72,63 and55) and three were described in rats. In humans, data on the metabolism of ecdysteroids is sparse and 3 metabolites were identified (69-70).



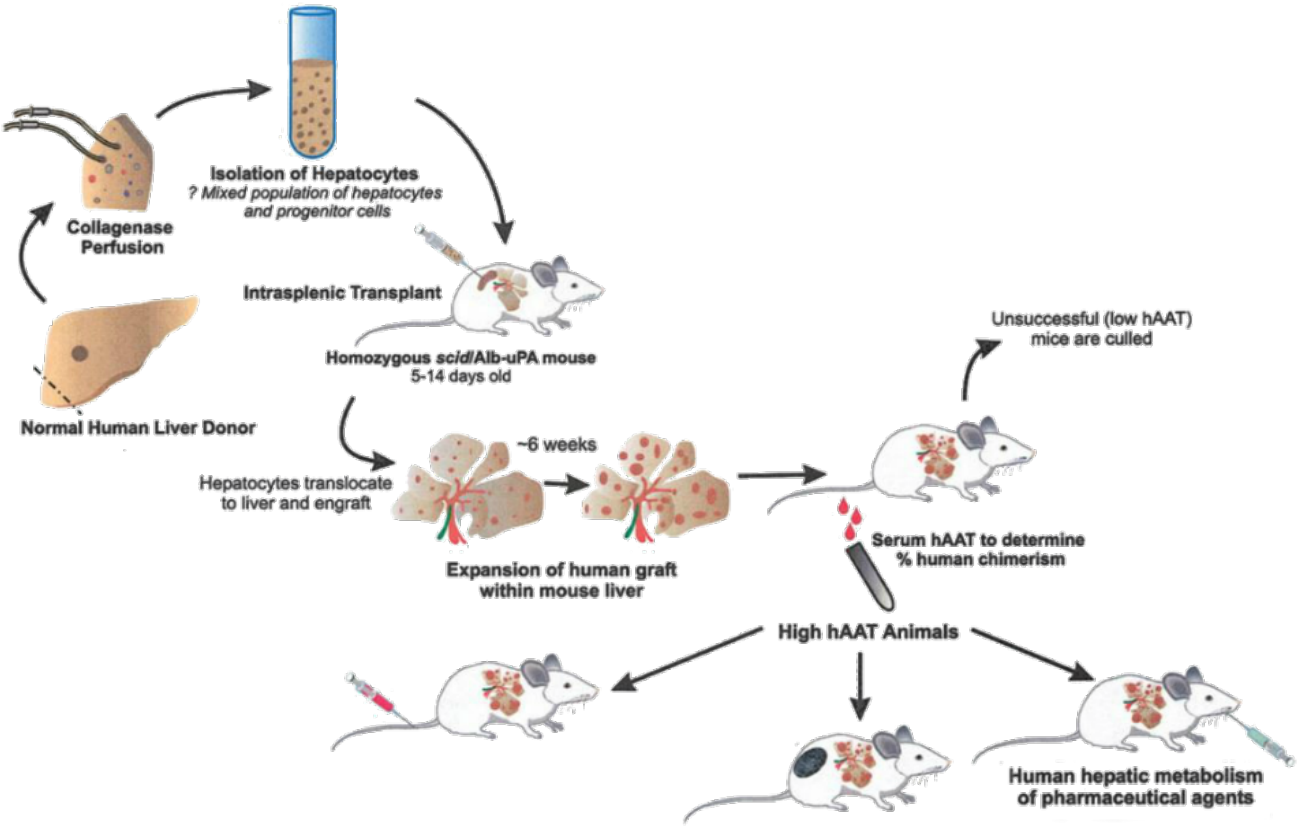
(Figure-6) Metabolism of injected 20-hydroxyecdysone in mice. Primary reactions producing three major metabolite species: a) 14-dehydroxylation; b) Complex reduction of the 7-en-6-one chromophore, and c) Epimerisation. Kumpun et al. (73).



(Figure-7) Metabolites of 20-hydroxyecdysone in human subjects. Following the administration of an oral dose of 20mg of 20HE to a human volunteer, 2-deoxy-20 hydroxyecdysone (a) and, 2-deoxyecdysone (b) were the two major metabolites identified, Tsitsimpikou et al. (69)

1.5. Models for the study of human hepatic steroid metabolism. Identifying whether the effects of various steroids/stimulants are exerted by the parent compound or its metabolites require investigation, ideally in humans. However, either ethical or legal considerations often make studies in humans difficult. Therefore, the challenge to identifying metabolites for the various AAS and other putative sport performance enhancing substances is the availability of an appropriate experimental model. Further, as the route, dosage and form of the steroids all become important considerations it has to a model that is readily available and reproducible. Most AAS and stimulants are taken as orally active substances, often over a period of time, and are predominantly metabolized by the liver. The use of the rodent model with the humanized liver may offer a good alternative to in vitro methods using human hepatocytes, which have been partially successful, but they lack the whole-body environment to generate a full complement of metabolites.

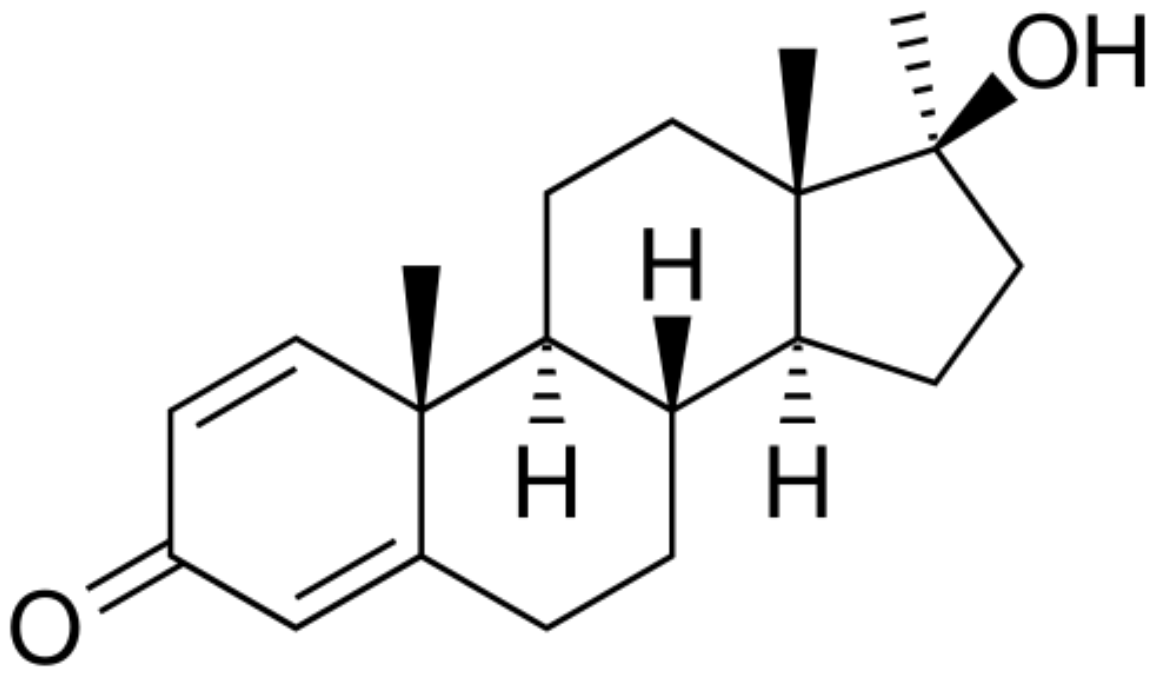
1.5.1. Murine models. Reports on chimeric mice with humanized livers achieved by transplantation of human hepatocytes into the spleen of various mice, such as the urokinase Plasminogen Activator-Severe combined Immunodeficient (uPA/SCID) mice, PXB-mouse or Fah/TK-NOG are available. All these mouse models have been used *in-vivo* studies to predict drug metabolism, pharmacokinetics and efficacy in humans (74-78). The uPA/SCID model was developed by Ghent University and is commercially available with KMT Hepatech Inc. (77) (Figure-8). In this model severe combined immunodeficient SCID mice, suffering from a transgene-induced liver disease are transplanted early after birth with primary human hepatocytes. The human hepatocytes integrate in the parenchyma and progressively repopulate the diseased mouse liver without losing their normal metabolic functions. These mice are called chimeric or "humanized" and the success of each mouse is measured by human albumin (hAlb) levels that correlates with human hepatocytes. It is claimed by KMT that the mice livers are up to 95% humanized. This model has been selected for the current project.



(Figure-8) SCID mice with humanized liver model. Soon after birth uPA+/+ SCID mice are injected with human hepatocytes in the spleen. The hepatocytes migrate and repopulate the mouse liver. Human alpha-1 antitrypsin is used as an indicator of successful engraftment.

1.5.2. Model Anabolic Androgenic Steroid. Methandienone (Figure-9), also marketed by Ciba as Dianabol, is an orally active, synthetic AAS. Daily doses have been reported as being between 20-25 mg, taken for 5 days per week, over a 5-week cycle. It has a half-life of 3.5 hours, with systemic peak levels being reached between 1.5 to 3 hours after ingestion. The primary urinary metabolite of methandienone has been shown to be detectable for up to 3 days, with more recent reports of the hydroxymethyl and sulphate metabolites in the urine for up to 19-26 days following a single oral (5mg) dose (79). Several of these metabolites are unique to methandienone. The metabolism of methandienone, and most other steroids that are orally administered, occur predominantly in the liver and excreted in the urine, hence the humanized liver SCID mice model may aid in the identification of the human hepatic metabolites from orally administered drugs.

As the metabolism of methandienone is so well documented it will be used as a model AAS to test the reproducibility and sensitivity of the chosen humanised murine model prior to embarking on studies on the metabolism of ecdysteroids.



(Figure-9) chemical structure of Methandienone; a gold standard doping agent widely used among Russian athletes in the 1970s.

1.6. Target organs of ecdysteroids.

Dietary intake and physical activity are key modulators of energy balance. The major peripheral tissues that dynamically participate in this are the adipose tissue and the skeletal muscle (80). Further, when considering sporting performance and factors that mediate this, the same tissues are likely to be salient targets. Exercise and training induce the mobilisation of fat mass, and specifically the breakdown of stored triglycerides and the release of free fatty acids (FFAs; 81). These FFAs act as fuel for the contracting skeletal muscle during exercise (82). The accumulation and depletion of fat in the adipose tissue impacts on mitochondrial and vascular activity, and factors that mediate this process are likely to impact on sporting performance (83). This is supported by intervention studies that shows the reduction of fat, and the build-up of lean (muscle), mass, in response to training and exercise (84). Adding ecdysteroids to this inter-organ crosstalk has yet to be investigated, especially considering that they are now proposed to be signalling via the ER. The role of mitochondria on these cell types of energetically active cells, adipocytes and skeletal muscle cells, has been shown to alter an organism's metabolic status.

1.7. Rationale for the study.

Despite the claims of ecdysteoirds, and mainly 20HE, having various beneficial effects on health and sport performance much of this is unproven, especially in humans, either *in-vitro* or *in-vivo*. Further, data on the metabolism of these compounds is also sparse, and the potential effect of the generated metabolites in mammals is unclear. Therefore, firstly, in order to even begin to understand their effects on humans, the metabolites of some of the most common of these ecdysteroids need to be investigated. Secondly, the effect of the ecdysteroids in humans is imperative especially since 20HE was added to the WADA monitoring list in October 2019 (Appendix 1). In order to investigate 20HE the following hypotheses were tested.

1.7.1 Hypotheses

1. 120HE can be detected in urine following ingestion of either multiple or single doses
2. The clearance of 20HE is associated with changes in the expression of several hepatic enzymes that regulate Phase 1 and Phase 2 metabolism.
3. Dietary supplement-derived 20HE, at doses too low to be anabolic, induce changes of blood pressure and mitochondrial membrane potential

1.7.2 Aims and objectives

1. Investigation of ecdysteroid metabolites.

a) Metabolites of methandienone.

The first part of the current study aimed to test the reproducibility and sensitivity of a humanized murine model (uPA(+/+)-SCID) to generate all the reported human metabolite species of methandienone and to investigate whether multiple dosing increases sensitivity of detection. Changes in expression of hepatic drug metabolizing enzymes, in mice treated by single or multiple doses were also determined, along with plasma aldehyde activity, in comparison to vehicle treated animals.

b) Metabolites of 20-hydroxyecdysone.

As the above model was found to be robust and able to reproduce previous data it has been used to study the metabolites of 20-hydroxyecdysone. One metabolite of 20HE was detected which has been confirmed as per previous reports. Several other metabolites were also apparent, and these will be investigated in future studies.

c) Human Excretion study

A single volunteer was recruited for the urinary excretion study of 20HE in its pure form and as constituent of a supplement.

2. Investigation of the human physiological effects of 20HE.

a) Human whole-body effects

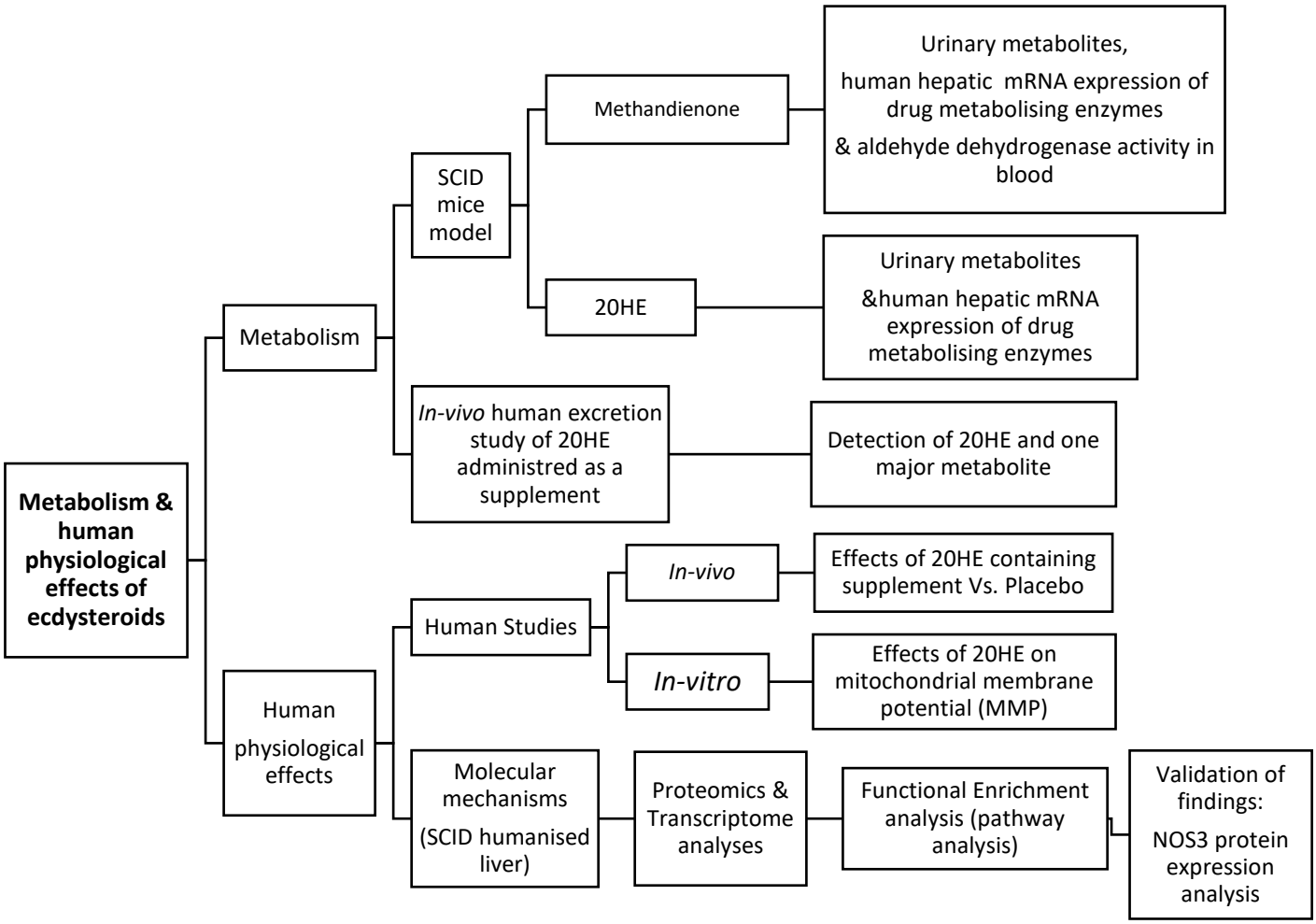
The hypothesis that supplement derived 20HE induced changes in body composition, blood flow and aerobic fitness was tested in human volunteers.

b) Effects of 20HE in *in-vitro* models

Effects of 20HE on mitochondrial function was studied utilising human preadipocytes and myoblasts.

C) An omics approach exploring the effects of 20HE on expression at the genes and proteins levels.

The overall schema for this thesis is shown in Figure-10.



(Figure-10) Schematic diagram representing the overall flow of the study. The project investigated two aims focused on the metabolism of 20HE and its physiological effects in humans respectively.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

Kits and reagents facilitating all studies were obtained from different vendors. Reagents and compounds used included: methanol, sodium hydrogen carbonate, potassium carbonate, RIPA buffer, acetic acid, ammonium bicarbonate, carbonilcyanide p-triflouromethoxyphenylhydrazone (FCCP), sodium hydroxide (NaOH), chloroform, isopropanol, sodium citrate, HEPES buffered physiological salt solution containing NaCl, KCl, MgSO4, KH2PO4, CaCl2, glucose, HEPES and media including: DMEM and Nutrient Mixture F-12 Ham were all obtained from Sigma Aldrich (St. Louis, Missouri, United States). Drugs injected into the murine models; Methandienone and 20HE, were obtained from Toronto Research Chemicals (North York, Ontario, Canada) and Sigma Aldrich respectively.

The two supplements chosen for the human *in-vivo* study; Desire-X and Z-force were obtained online through Amazon.com (USA), from Manson vitamins and Dymatize nutrition respectively. The sources of the remaining 14 supplements analysed are summarised in (Table-3). Other reagents used in the *in-vivo* studies included: β-glucuronidase from E. coli; Roche Diagnostics (Basel, Switzerland), Diethyl ether; Merck (Kenilworth, New Jersey, United States), MSTFA; Carl Bucherer (Lucerne,Switzerland), 2-propanthiol; Merck, Iodoacetamide; Biorad (Hercules, California, United States), ammonium formate; Agilent Technologies (Santa Clara, California, United States) and LC Grade acetonitrile & 1,4-dithiothreitol (DTT) were both obtained from Merck.

Reagents for the extraction and processing of DNA, RNA and protein were obtained from various vendors; Ethanol; Ciba-Chem (Basel, Switzerland), Phosphate Buffered Saline (PBS); Gibco (Waltham, Massachusetts, United States), DEPC water &TRIzol reagent; Ambion (Austin, Texas, United States) and Formic acid; Merck. Kits used were GeneChip WT PLUS Reagent Kit & High-Capacity cDNA Reverse Transcription kit; Applied Biosystems (Foster City, California, United States), Pierce BCA protein assay kit; Thermo Scientific (Waltham, Massachusetts, United States) and Trypsin/Lys-C Mix, Mass Spec Grade V5072; Promega (Madison, Wisconsin, United States). β-actin Primer Assays, first strand cDNA synthesis kit, Pathway specific: Human/Mouse drug Metabolism: Phase I Enzymes, Human Drug Metabolism PCR Array and ABI SYBR Green master mix were all obtained from Qiagen (Hilden, Germany). Finally, ALDH Activity Assay Kit was obtained from BioVision (Milpitas, California, USA).

Materials used for the validation study included cryopreserved Human Coronary Artery Endothelial Cells (HCAECs), Endothelial Cell Growth Media and supplement pack and were all obtained from Promocell (Heidelberg, Germany) while attachment factor was obtained from Gibco. SeeBlue plus2 protein standard marker was obtained from Invitrogen, mouse monoclonal anti-eNOS antibody (M221) purchased from Abcam and eNOS QuantiTect primer assay was obtained from Qiagen.

Name	Origin	Website (seller)	Duration till delivery	Instructions at delivery
Turkesterone	Thailand	S5 supplement labs	14 days	None
Ecdybolin	Russia	eBay (Truly huge supplements)	7 days	None
Suma root	USA	eBay (Swanson health products)	6 days	None
Desire-X	USA	Amazon (Manson vitamins)	12 days	None
Immunectar	USA	eBay (Natural organics laboratories, inc)	8 days	None
Natural Sterol extreme	UK	eBay (Universal nutrition)	10 days	Declaration form
Medicinal cyathula root	China	Made in China (Shaanxi Sinuote Biotech Co. Ltd)	7 days	None
ZMA PM	USA	eBay (Kaizen nutrition)	14 days	None
Beta-X	USA	Bonanza (Mass nutrition)	10 days	None
Norateen II	UK	eBay (LA muscle)	7 days	None
Z-Force	USA	Amazon (Dymatize nutrition)	14 days	None
Bio pro plus	USA	Amazon (Alternative health concepts)	12 days	None
MSB (Methyl plus 4th Generation)	Canada	Nutrichem	20 days	Declaration form
Power meal	USA	eBay (Young living essential oils, LC)	14 days	None
Animal Stack (USA)	USA	eBay (Universal nutrition)	20 days	Declaration form
Promax Extreme	UK	eBay (Maxi nutrition (GSK healthcare)	15 days	None

(Table-3) Origin, seller sources and shipping details of the 16 supplements analysed for the human *in-vivo* study to investigate their ecdysteroids content. Out of 149 supplements and preparations claiming the content of ecdysteroids reported by Lafont et. al. (55) only 16 were obtainable due to different reasons including product discontinuation.

Cells, media and reagents used for the measurement of mitochondrial membrane potential were obtained as follows; Human White Preadipocytes (HWP) from the subcutaneous adipose tissue depots of normal weight subjects Cat no: C-12730, Lot no: 395Z024 were obtained from Promocell and HSMM-Muscle Myoblasts, basal media, SkGM™ SingleQuots supplement pack were all obtained from Lonza (Basel, Switzerland). Tetramethyl rhodamine methyl ester (TMRE) for the staining of the mitochondria was obtained from Invitrogen (Carlsbad, California, United States).

A wide range of instruments were used in these studies. The instruments used for the extractions, quality assessment and downstream analyses of DNA, RNA and protein included GLS Aqua 12 Plus water bath & QBD2 Heat block; Grant (Cambrigeshire,UK), Dounce all glass tissue grinder; Kimble Chase (Vineland, New Jersey, USA) Genius 3 Vortex; IKA (Staufen, Germany), 5424 R Centrifuge; Eppendorf (Hamburg, Germany), SL16R Centrifuge and SeriesII water Jacket Co2 incubator both from Thermo Scientific (Waltham, Massachusetts, United States) in addition to the quality assessment instrument; NanoDrop while the Bioanalyzer was obtained from Agilent .The ViiA7; Applied Biosystems (Foster City, California, United States) and the Infinite 200 PRO plate reader; Tecan (Männedorf, Switzerland). For the transcriptome analysis the GeneTitan instrument was utilised; Affymetrix (Santa Clara, California, United States).

For the detection of urinary metabolites and the proteomics analysis these instruments were used: UHPLC, QExactive Orbitrap-based mass spectrometer and ELITE Orbitrap LC/MSMS from Thermo Scientific and Zorbax Eclipse Plus C18 column, GC 7890 system, 7000C triple quadrupole mass spectrometer from Agilent technologies in addition to LabRoller Lab Rotator mixer from Thomas Scientific (Swedesboro, New Jersey, United States).

For the human anthropometric measurements and body composition electrical bioimpedance MC 980MA TANITA (Tokyo, Japan) was used. Quark CPET Cosmed (Rome, Italy) was used for the assessment on aerobic fitness and physiological responses to exercise. Haematology tests and clinical chemistry assays were conducted on the XT4000i instrument from Sysmex (Kobe, Japan) and the Roche Cobas integra 400 plus; Roche (Basel, Switzerland) respectively. Finally, Leica confocal microscopy (Wetzlar, Germany) was utilised for the mitochondrial membrane potential measurements.

Software used for the statistical analyses were Microsoft Excel; Microsoft (Redmond, Washington, United States), SA Biosciences online data analysis / Gene Globe Data Analysis Centre; QIAGEN (Hilden, Germany), Transcriptome Analysis Console TAC Version 4.0; Affymetrix (Santa Clara, California, United States), Raw Meat proteomics data analysis software, Proteome Discoverer PD Thermo scientific. Web-based data analysis tools were also used for functional enrichment analysis on gene and protein lists exported from the transcriptome and proteome analyses included STRING version 11.0 (91), g:Profiler (92)and freely available to download software FUNRICH VERSION3.1.3.

Murine Studies. All the mouse *in-vivo* experiments were conducted at KMT Hepatech lnc. (Edmonton, Canada) and approved by the Laboratory Animal Ethics Committee. It is noteworthy that in 2017 KMT Hepatech became part of PhoenixBio group.

Human Studies. The study was approved by Anti-doping lab institutional review board (Ref. F2018000296) and conducted in the licensed clinic of ADLQ, which was fully equipped to facilitate all the later described protocols with minimal risk to the participants.

2.2Methods

Metabolites and excretion profiles studies have mainly been conducted *in-vitro* using human hepatocytes. Often such studies don't provide similar results to those conducted *in-vivo* in clinical studies. It is known that clinical *in-vivo* investigations are governed by strict ethical guidelines hence, to investigate physiological effects of 20HE or any other substance, a suitable *in-vitro* and/or *in-vivo* models were sought. The need for an alternate model was addressed by Lootens et. al. (76) where their study examined the use of uPA+/+ SCID mouse model for such investigations. The initial aim was to confirm the results obtained by Lootens et.al (76) on the metabolism of the gold standard doping agent, Methandienone. This study aimed to validate the use of the model at ADLQ facilities, with the *in-vivo* portion of the experiment conducted at KMT facilities in Edmonton, Canada. After confirming the detection of the human hepatic metabolites of this doping agent, the second part aimed to investigate the metabolism of 20HE using the same uPA+/+SCID mouse model after its suitability was confirmed. 20HE excretion was investigated using urine samples obtained from the SCID mice and in a single human volunteer administered with 20HE in its pure form and as a constituent in a supplement. These studies were conducted using one common route of drug excretion which is the urinary route.

Although human *in-vivo* models offer the best results they are very challenging due to ethical restrictions. In this study specifically, 20HE has been reported to have, in addition to the anabolic activity, side effects that can be considered threat to health, including non-specific organ hypertrophy (54). This created an ethical issue that compromised any human *in-vivo* study where 20HE is administered in its pure form. Though the uPA+/+ SCID mouse model explored in the first aim was one way of addressing this issue, it came with many limitations since it was only the human liver within the model that can be studied. This excluded the ability to look at any systemic effects due to the compromised nature of the model. In the following study, the ethical issue of *in-vivo* administration of a drug was addressed in a different manner than in the first study, which utilised the

mouse model with humanised liver, by the investigating the effects of 20HE *in-vivo* in humans by administering a specific concentration of 20HE as a component of a supplement that was readily available in the market. Sixteen supplements were obtained from different sources claiming ecdysteroid content and then were analysed to quantify 20HE concentration (Table-3). Two supplements were chosen for the human study based

on their ecdysteroid content concentrations. The first supplement; Desire-X consisted of 20HE concentration that was lower than the reported anabolic concentration. The second supplement (Z-Force) had undetectable amounts of 20HE and was used as a control. Supplements intervention was given to healthy recreational athletes for two months and before-and-after parameters were measured, recorded and analysed.

Literature showed the anabolic effects of 20HE in muscles in rats and mice (66,73) and anecdotal reports indicated the reduction of fat mass in response to 20HE. To further investigate this the effects of 20HE on mitochondria *in-vitro* in Human Skeletal Muscle Myoblasts (HSMM) and Preadipocytes was investigated. In mitochondrial respiration, energy stored in micronutrients is converted to ATP which is the cellular energy donor. This is done by the oxidation of micronutrients coupled with ATP synthesis (94). In this study confocal microscopy was utilised to measure the coupling of mitochondria after treatment with different concentrations of 20HE.

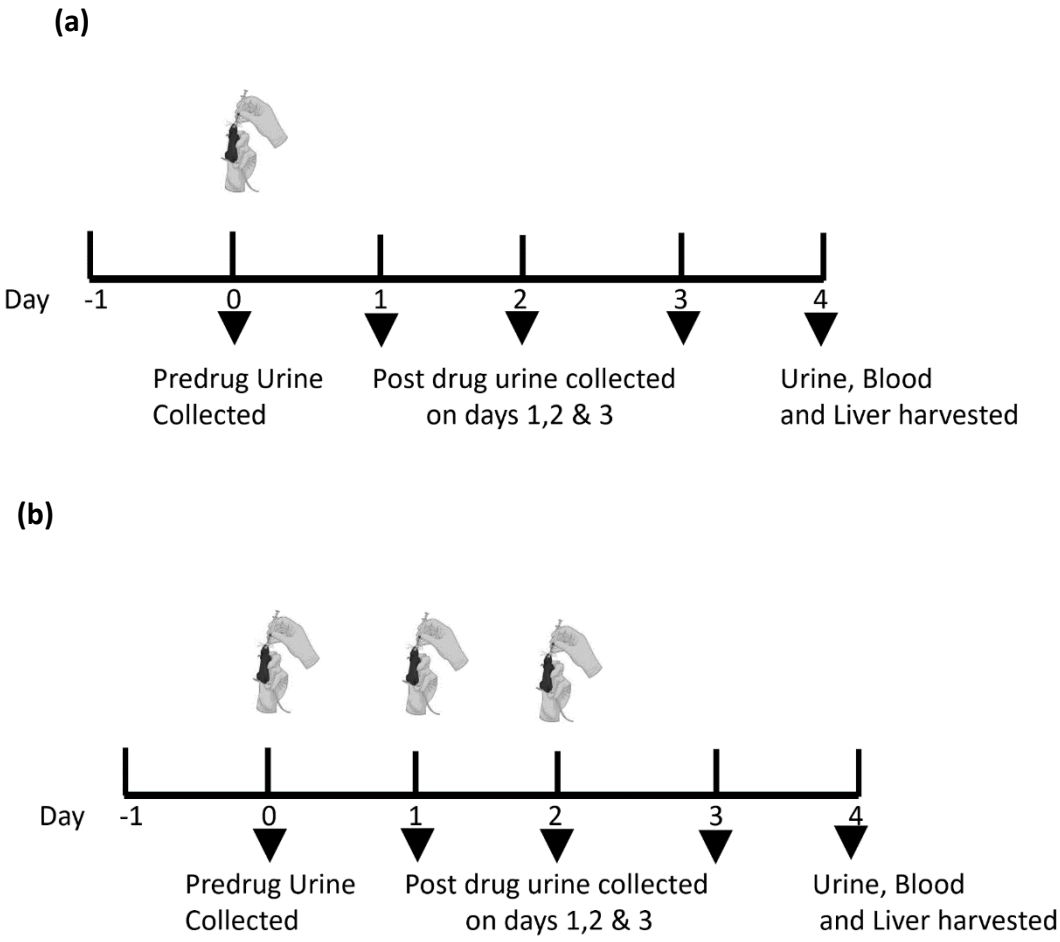
Finally, molecular mechanisms relating to metabolism and physiological effects of 20HE were investigated. This was done at the RNA and protein level extracted from the humanised liver SCID mice model treated with 20HE. Whole transcriptome and proteome approaches were used, and data analysis was conducted in-house and taken further with collaboration with Qatar Computational Research Institute QCRI.

2.2.1 Investigation of 20HE metabolism

2.2.1.1 Metabolism of Methandienone

Animal study

All *in-vivo* mouse experiments and procedures were approved by the Laboratory Animal Ethics Committee and were carried out at KMT Hepatech Inc. uPA+/+ SCID mice were transplanted with primary human hepatocytes (chimeric mice). Non-chimeric uPA+/+ SCID mice without transplanted human hepatocytes were used as controls. Prior to administration of the drug, human albumin concentrations were determined in the plasma of all mice. Chimeric mice with elevated human albumin were used in the study.



(Figure-11) Methandienone/placebo-treated uPA+/+ SCID mice experimental design and sample collection. Mice with humanised liver (chimeric) were administered with drug (n=3) or placebo (n=3) in a single dose (a) similar experiment were conducted as a control on non-chimeric mice administered with drug (n=2) or placebo (n=2). Another experiment design was conducted with multiple dosing (b) where chimeric (n=6) and non-chimeric (n=4) mice were administered with either the drug (n=3) and (n=2) respectively or placebo (n=3) and (n=2) respectively. Urine samples were collected every 24 hours and on day4 animals were sacrificed. Urine and blood samples were collected, and livers were harvested.

The chimeric mice which were successfully transplanted with human liver (n=12) were divided to two groups. The first group (n=6) was administered with (100µl of 400mg/L) of methandienone in a single dose (n=3) or multiple doses (n=3). The second control group (n=6) was administered with placebo; 5% ethanol/PBS in single dose (n=3) or multiple doses (n=3). Drug and placebo administrations were done by oral gavage. Similarly, non-chimeric mice were divided in to two groups. The first group (n=4) was treated with methandienone in single dose (n=2) or multiple doses (n=2) while the second control group (n=4) was administered with placebo; 5%ethanol/PBS in a single dose (n=2) or multiple doses (n=2). Methandienone was administered by oral gavage on day 0 for the single dose group (Figure-11a), and on days 0, 1 and 2 of the experiment for the multiple doses group (Figure-11b).

Urine samples from all groups were collected, noninvasively over a 24h period, on days 0 prior to the drug administration and on days 1, 2, 3 and 4 after the drug administration. All samples were then stored at -20°C. A total of 20 mice were used in this study (Table-4). All animals were sacrificed by Schedule 1 protocol at the end of the experiment. Blood plasma was obtained by cardiac puncture with anticoagulant following which the livers were harvested. Livers were flash frozen and samples (urine, plasma and liver tissue) were stored at -80°C until transported to ADLQ on dry ice for analysis.

Urinary metabolites analysis

All urine samples collected from the uPA+/+ SCID mice were analysed by Ultra-Performance Liquid Chromatography–High-Resolution Mass Spectrometry (UHPLC/HRMS) and Gas chromatography mass spectrometry (GCMS) for the detection of the parent drug and its major metabolites. Additionally, a blank human urine sample fortified with reference standards of the previously reported metabolites was analysed to confirm the presence of the detected metabolites.

Hydrolysis

In a glass tube 400µL of urine was mixed with 75µL of ß-Glucuronidase enzyme 20KU/141mL, 500µL Phosphate buffer (pH7) and 25µL on internal standard consisting of a mixture of 6 steroids. The tube was then sealed and vortexed for few seconds and incubated in the oven at 55^{o}C for 1hour after which it was cooled for 5-10 minutes. Glucuronidase enzyme cleaves the sugar group (COOH) in all the OH groups which only works at pH7 and the Phosphate buffer fixes the pH at 7.

Liquid-Liquid Extraction

After hydrolysis, one spoon (≈ 0.5g) of $NaHCO_3$ and 5mL Diethyl ether were added. The tube was again sealed and vortexed for few seconds. Hydolysis caused ionization and the addition of $NaHCO_3$ increased the pH to 10 deionizing compounds of interest. The tube was then placed on a LabRoller rotator to mix at a moderate speed for 20 minutes to separate the organic phase from the aqueous phase. After the incubation the tube was centrifuged at 3000RPM for 12 minutes and placed in a -80^oC freezer in a rack filled with ethanol to speed up the freezing process. Compounds of interest then separate into the organic phase in ether -which doesn't freeze at -80^oC- from the frozen solid aqueous phase. The organic phase was then collected in a new falcon glass tube and the sample was evaporated in a heat block at 55^oC with nitrogen flow until fully dry. After this LC and GC analyses protocol differ where for LC analysis, 100 µL reconstitution buffer (80%A|20%B) was added and sample was transferred to injection Vials. And for GC analysis derivatisation process is done to the dried sample where 50µl of derivatisation reagent consisting of 100mlMSTFA + 400mg NH_4I+300µL 2-Propanthiol was added to the tube and incubated on a heat block at 55^{o}c for 1 hour. The functions of the constituents of the derivatisation mixture were; NH_4I for the removal of H+ from the compounds of interest, MSTFA used for Silylation process that involve replacing H+ in compounds of interest and finally 2-Propoanthiol was used to react with the excess NH_4I. The samples were then transferred to injection vial of GCMS instrument Agilent GC 7890 coupled with an Agilent 7000C QQQ MS, equipped with an Agilent 7693 auto-sampler.

UHPLC/HRMS conditions were as follows; the Dionex UHPLC system was used for the chromatographic separation according to established method in the anti-doping lab. This system consists of a vacuum degasser, a high-pressure binary pump, an autosampler with a cooled sample tray and a column oven. Chromatographic separation was performed at 40°C using a Zorbax Eclipse Plus C18 column (100 x 2.1mm i.d., 1.8 mm particle size). The mobile phase consisted of 5mM ammonium formate in 0.02% formic acid (solvent A) and a mixture of acetonitrile/water (90:10 v/v) containing 5mM ammonium formate and 0.02% formic acid (solvent B). A gradient elution program was employed at a constant flow rate of 0.2 mL min-1. The analysis run time was 20 minutes and the injection volume was 5µL.The high-resolution mass spectrometry was performed using a QExactive Orbitrap-based mass spectrometer. GC/MSMS analysis was performed using Agilent GC 7890 system coupled with Agilent 7000C triple quadrupole mass spectrometer equipped with a 7693 autosampler and chromatographic separation was achieved using a SGE BPX5 column. The MS system was a QQQ with Electron Ionization at 70eV and multiple reaction monitoring (MRM) acquisition for the detection of methandienone and its metabolites.

Study Group	Animal ID #	Treatment	Animal ID #	Treatment
Group 1 (Chimeric)	S035	Single Dose /Drug	S049	Multiple Doses/Drug
	S140		S053	
	S143		S356	
Group 2 (Chimeric)	R909	Single Dose /Placebo	S050	Multiple Doses/Placebo
	S034		S052	
	S036		S355	
Group 1 (non-chimeric)	R701	Single Dose /Placebo	S460	Multiple Dose/Drug
	R704		M-A218	
Group 2 (non-chimeric)	R700	Single Dose /Drug	S458	Multiple Dose/Placebo
	R711		M-A216	

(Table-4) Animals allocated to single and multiple doses of Methandienone. Eight chimeric uPA+/+ SCID mice were treated with either Methandienone or placebo (placebo) in single dose (left) or in multiple doses (right). The same was conducted on non-chimeric mice serving as controls.

Microarray analysis

To extract the RNA, liver tissue harvested from the experimental animals was placed on ice and kept frozen by frequent exposure to liquid nitrogen while grinding the tissue to fine powder using pre-chilled mortar and pestle. TRIzol reagent was added to disrupt the cells for complete cell lysis. RNA was then precipitated with isopropanol; the pellets were washed with 70% ethanol and after airdrying, the pellet was resuspended in Nanopure DEPC water and stored at -80^{o}C until further analysis. RNA quality was checked using the NanoDrop and the Bioanalyzer where RNA Integrity Number (RIN) of 5.5 was considered acceptable for expression studies. cDNA was synthesised using Qiagen first strand cDNA synthesis kit. To eliminate genomic DNA contamination, RNA samples were incubated with GE buffer for 5 minutes at 42°C, samples were then placed immediately on ice for at least 1 min. Reverse transcription master mix was then prepared for all samples which consisted of the buffer, control and reverse transcriptase enzyme. The mix was then added to all RNA samples and incubated at 42°C for exactly 15min. The reaction was immediately stopped by incubation at 95°C for 5 min. RNase free water was then added to each sample. For pathway specific microarray analysis cDNA was added to SYBR green mixture and loaded into the 96 well plate array. The array was then sealed and centrifuged for 1 minute at 1000 g at room temperature to collect the contents to the bottom. The array was then loaded into the ViiA7 real time PCR instrument with these cycling conditions: activation by heating at 95°C for 10 minutes, annealing and elongation at 95°C for 15 seconds and 65 °C for1 minutes respectively for 40 cycles and finally fluorescence data were collected. This protocol was applied for the human and mouse Drug Metabolism: Phase I Enzymes PCR Arrays to assess the expression of 84 key Phase 1 drug metabolism genes by real time PCR (appendix-2). Endogenous reference RNA controls were used to normalise the amount of target gene for relative quantification. Human and mouse arrays were used according to manufacturer recommendations.

Aldehyde dehydrogenase activity in blood

The aldehyde dehydrogenase activity in plasma was investigated in response to methandienone using the ALDH Activity Assay Kit (BioVision's PicoProbe). The assay was conducted on Infinite 200 PRO Microplate Reader from Tecan according to the manufacturer's protocol in kinetic mode. The kinetic cycle was of 2-minute interval for 1 hour. Fluorescence intensity was measured from the bottom of the plate at Ex/Em 535/587 nm. The reading was recorded after 10 flashes with manual gain of 100.

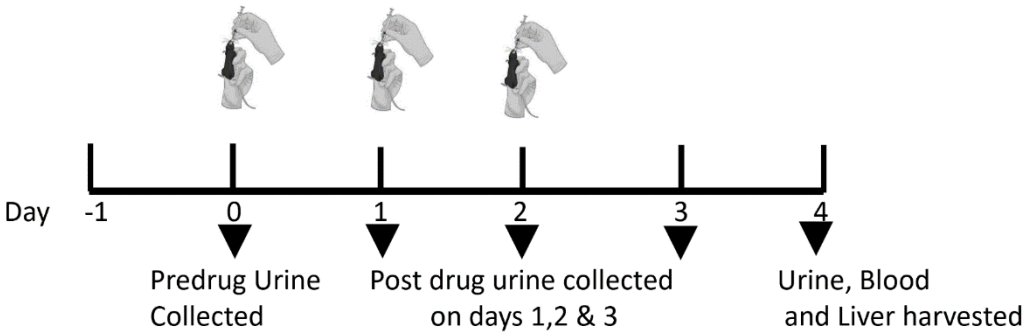
The ALDH activity=B (ΔT x V) x sample dilution factor = pmol/min/mL = μU/ml.

Where: B is the amount of NADH generated in the sample (pmol), ΔT is the reaction time and V is the sample volume.

2.2.1.2 Metabolism of 20HE

Animal study

uPA+/+ SCID mice were transplanted with primary human hepatocytes and human albumin concentration was determined prior to drug administration to confirm the engraftment of human hepatocytes. Non-chimeric mice served as control animals. Chimeric mice (n=8) were administered with multiple doses of 20HE (n=4) or 5% ethanol/PBS placebo (n=4). Similarly, non-chimeric mice (n=2) were also administered with 20HE (n=1) or 5% ethanol/PBS placebo (n=1). 20HE was administered by oral gavage at a concentration of 200μg/gavage (100μl of 2.0mg/ml) on day 0, 1 and 2 of the experiment. Metabolic cages were used to collect urine samples noninvasively from all animals on days 0,1,2,3 and 4 of the experiment and stored at -20°C. All animals were sacrificed by Schedule 1 protocol at the end of the experiment (Figure-12). Blood was obtained by cardiac puncture with anticoagulant using EDTA tubes. Livers were harvested and flash frozen. Samples (urine, plasma, and livers) were stored at -80°C until analysis.



(Figure-12) 20HE\placebo treated uPA +/+ SCID mice experimental design and sample collection. Male chimeric (n=8), non-chimeric (n=2) uPA+/+ SCID administered with 200µg/gavage 20HE or placebo by oral gavage, in multiple doses. 24h urine collections were made prior to and daily following drug administration for up to 4 days. On day 4 animals were sacrificed and urine and blood were collected, and livers were harvested.

Urinary metabolites analysis

Gas Chromatography analysis started with addition of 1mL of phosphate buffer at pH7 (0.8M K_2HPO_4 and 0.4M KH_2PO_4), 50µL of β-glucuronidase from E. coli and 50µL of a methanolic solution of internal standards mixture to 0.5 mL of urine sample. The mixture was then hydrolysed by incubation at 50°C for 1.5 hours. After hydrolysis, the pH was adjusted to 9–10 with sodium hydrogen carbonate and potassium carbonate (2:1) (w/w). A liquid–liquid extraction against 5mL of diethyl ether was performed. After centrifugation, the organic layer was separated from the aqueous phase by freezing and evaporation under nitrogen at 50°C. Prior to GC/MSMS analysis, a derivatisation step was performed to the dry residue by adding 25µL of MSTFA/Ammonium iodide/2-Propanthiol (500:2:4) followed by incubation for 1 hour at 100°C.

Microarray analysis

RNA extraction was carried out as detailed previously by grinding the frozen tissue in liquid nitrogen. TRIzol was added to disrupt the cells and RNA was precipitated using alcohol precipitation method. The quality was checked using a Bioanalyzer and NanoDrop. cDNA was synthesised and used for pathway-specific human drug metabolism PCR arrays (Qiagen) to assess the expression of 84 key drug metabolism genes by real time PCR as described in previous section.

2.2.1.3 Human Excretion study

To conduct excretion studies, the quality and quantification of 20HE sources must be analysed. So, 16 supplements that claimed having ecdysteroid as a component were analysed by GCMS. Based on the results from this quantitation the supplement containing 20HE and the placebo supplement were chosen for the excretion studies.

Supplement analysis

The extraction step started with dissolving each capsule by shaking in 5mL of carbonate buffer at pH 10-11 for 30 minutes. Then, 5mL diethyl ether was added and samples were incubated for another 30 minutes with shaking. The mixture was then centrifuged at 3500 RPM for 10 minutes to separate the organic layer, which was evaporated under nitrogen flow. The pellet was reconstituted in 200µl of mobile phase (100% water containing 5mM ammonium formate in 0.02% formic acid) and analysed using GCMS to detect 20HE quantitatively. Interestingly supplement quantification showed that Turkesterone supplement was mislabelled and contained 2.3mg of 20HE.

Excretion study

The excretion study started with the recruitment of one healthy 43 years old volunteer weighing 90Kgs who declared not to have used any nutritional supplements or medications during the last 3 months. A blank urine was collected and a single 2-tablet dose of Turkesterone supplement (recommended dose by the manufacturer) was administered. Urine samples were collected before taking the supplement on day one and continued after taking the supplements for a total of 4 days. After a 45-days washout period, the same volunteer was again recruited and administered with a single dose Desire-X supplement which after quantification yielded a lower concentration of 20HE compared to Turkesterone supplement. The dose consisted of 2 tablets taken on day one and urine samples were also collected for 6 days thereafter. All the urine samples were stored frozen at -20°C until analysis by GCMS.

This concluded the methods relating to the investigation of 20HE metabolism. The 16 supplements that were analysed by GCMS revealed ranging amounts of 20HE concentrations within each supplement and this intrigued further exploring of the possible physiological effects in humans *in-vitro* and *in-vivo*. As higher concentrations of 20HE were reported to be anabolic in mammals the following investigation aimed to examine the possible physiological effects of 20HE at lower concentrations.

2.2.2 Physiological effects of Ecdysteroids

2.2.2.1 Human *in-vivo* study

A cohort of non-diabetic normotensive recreational athletes with no history of supplement intake for the previous 3 months was recruited by word of mouth (Table-5). The criteria for inclusion or exclusion from the study is summarised in (Appendix-3). Volunteers who passed the inclusion criteria were divided to two groups; group 1 received the 20HE containing supplement Desire-X (n=6) and group 2 received Z-force as a placebo supplement (n=6). During the first visit all participants signed an informed consent form (appendix-4) and completed a questionnaire detailing their dietary intake and training schedule and co-morbidities on the day of study. After that baseline measurements were recorded including anthropometric measurements; body mass index – body weight (kg) in light clothing and height (m) were recorded. Body composition analysis was conducted using bioimpedance Tanita MC980MA. Systolic (SBP) and diastolic blood pressure (DBP) were recorded using automatic digital blood pressure monitor and then mean arterial BP MAP) was calculated. Multiple urine samples were collected from participants taking Desire-X (20HE) before and during the intervention to ensure compliance by taking the supplement. All groups were residing in Doha, Qatar for the whole duration of the study period.

Phase	Time of visit / duration	Procedure
1: Screening and intervention	Morning/ 4-6 hours	**Screening in a fasting state:** Questionnaire, Body Composition, anthropometric measurements, BP, CPET aerobic fitness, Blood sampling and urine collection. **Intervention:** Volunteers were asked to start the use of the supplement for two months
2:Post intervention	Morning/ 4-6 hours	**Post intervention screening:** Body Composition, anthropometric measurements, BP, CPET aerobic fitness, blood sampling and urine collection
3:Data acquisition and analysis	–	Blood was tested using Sysmex (haematology tests) and Cobas integra 4000 (chemistry tests). Urines were analysed by GCMSMS for the detection of metabolites. Data were analysed using SPSS.

(Table-5) Human *in-vivo* study Scheme consisting of three phases. Phase1: Screening and baseline measurements recording then the start if the intervention, Phase 2: Post intervention measurements recording and phase3 data analysis.

To investigate the effects on aerobic fitness, Bruce protocol was used before and after supplement intervention with or without 20HE using CPET (Cardio pulmonary Exercise Testing). The speed and angle/grade of the treadmill was set to increase at 3-minute interval beginning at 10% grade and 2 .7 Km/h for 3-minutes and gradual increase up to 22% grade and 9.6 Km/h (Table-6) where VO_2 association with the heart rate induced and then the VO_{2max} is automatically calculated.

Total blood extraction was 12mL at one visit divided into the following tubes with and without anticoagulant (3mL each), NF (sodium fluoride) 1mL tube and PAX gene Blood RNA tube. Blood Glucose, Total Cholesterol, LDL-Cholesterol, HDL-Cholesterol levels and Triglycerides levels were measured using Cobas integra 400 plus and Sysmex was used for haematology tests. Collected urine samples from the 20HE supplement group were analysed using GCMSMS. Sample preparation, hydrolysis and derivatisation was done according to the protocols detailed in section 2.2.1. Finally, advice on a balanced healthy diet was provided to all volunteers both in a face –to –face interview and in the form of printed material. All volunteers were asked to keep a food diary for 3 days; two workdays and one day in a weekend for the assessment of quality and quantity of dietary intake. Compliance to the diet was also monitored by the information provided in the food diary.

Stage	Minutes	% grade	km/h	METS
1	3	10	2.7	5
2	3	12	4.0	7
3	3	14	5.4	10
4	3	16	6.7	13
5	3	18	8.0	15
6	3	20	8.8	18
7	3	22	9.6	20

(Table-6). Bruce protocol conducted using CPET for the estimation of cardiorespiratory fitness (aerobic fitness). Each stage is held for 3 minutes and grade% which refer to the treadmill angle is increased by 2% after each stage with increased speed at every stage. METS represents the consumption volume of oxygen during rest.

2.2.2.2 Human *in-vitro* studies

Preadipocytes Mitochondrial membrane potential

Primary human white preadipocytes from the abdominal subcutaneous and visceral adipose tissue depots were obtained from non-diabetic female and male subjects respectively and their characteristics are detailed in (Table-7). Cryopreserved cell pellets were resurrected in the recommended growth media and grown as per manufacturer's recommendations. The resurrection was successful, and the cells expanded over two generations (i.e. passaged twice; P2). The P2 cells were counted and 30,000 cells in 1mL of media was used for the following studies. Cells were cultured until approximately 80% confluence on polystyrene tissue culture plates.

To measure mitochondrial membrane potential ($\Delta\Psi_m$ MMP) initially two thousand cells from each preadipocyte depots were seeded on two glass coverslips each and were allowed to adhere onto the matrix overnight, at 37^{o}C and 5% CO_2. One coverslip per cell type was treated with 10µM of 20HE and one acted as control (without treatment), both were incubated overnight at 37^{o}C with 5% CO_2. Dose response studies were then conducted where four coverslips comprised one experiment consisting of 0 ,1, 5 & 10 µM 20HE treatments. After 24 hours of treatment cells were washed with HEPES buffered physiological salt solution containing 156mM NaCl, 3mM KCl, 2mM $MgSO_4$, 1.25mM KH_2PO_4, 2mM $CaCl_2$, 10mM glucose and 10mM HEPES, and pH was adjusted to 7.4 with NaOH. After three washes the cells were incubated in 30 nM tetramethyl rhodamine methyl ester (TMRE) for 30 minutes to stain the mitochondria. TMRE is a lipophilic cationic dye that equilibrates between compartments in response to potential differences. TMRE was used at very low concentrations (30 nM), when the signal is proportional to dye concentration, which in turn depends on the potential differences between compartments, so that comparative estimates of $\Delta\Psi$ may be obtained from confocal images. After the incubation, TMRE fluorescence was excited using a single wavelength of 543 nm HeNe laser line and measured at >590 nm using Leica confocal microscope. A decrease in TMRE signal indicates mitochondrial membrane depolarisation. Quantitative measurements of TMRE intensity per field was made using the inbuilt software. Further these parameters were measured following uncoupling with carbonilcyanide p-triflouromethoxyphenylhydrazone (FCCP).

RNA and cellular protein extraction

Cells of the same batch and passage number were used in these experiments so that mRNA and protein expression were truly representative of those that had mitochondrial function measured. The cells were grown in 6-well plates to confluence before being treated with 20HE overnight. The control cells were treated with media only, minus the 20HE. The media was decanted, and the plates kept on ice throughout. The cells were washed once with ice cold PBS and any excess removed. RNA was extracted with TRIzol from these cells as previously described.

Extraction of cellular protein using RIPA buffer

The cellular protein was extracted by adding 500 µl of RIPA Lysis Buffer with inhibitors to each well and shaken from side to side to distribute evenly. The cells were scraped off the plate and the lysate transferred to a 15 mL conical tube. The lysate was vortexed vigorously for 5 minutes for three times and then incubated on ice for 15 minutes before being centrifuged at 13,000g for 5 minutes at 4°C. The supernatant was collected into fresh 1.5mL Eppendorf tubes, without disturbing the pellet. The protein concentration was estimated on the Nanodrop. Aliquots were stored at -20°C until analysis.

Product Name	**HWP**	**HWP**
Order Number	C-12732 / C-12733	C-12730 / C-12731
Lot Number	410Z041.2	419Z023
Donor Age / Sex / Race	56 / Male / Caucasian	45 / Female / Caucasian
Tissue / Localisation	visceral adipose tissue / omentum	subcutaneous adipose tissue / abdomen
Number of Viable Cells	600.000	600.000
Stage of culture	shipped in passage 2 (3rd culture)	shipped in passage 2 (3rd culture)

(Table-7) Characteristics of primary visceral and subcutaneous human white preadipocytes (HWP). Details of the cells used in the first set of experiments conducted on the confocal microscopy for the investigation of mitochondrial membrane potential are shown in the table.

Test method for combined DNA, RNA and Protein extraction from treated cells

In addition to the conventional method used for RNA and protein extraction from cells, a combined procedure described here was attempted**.** 500µl of Trizol reagent was added to each well containing cells and incubated on ice for 5minutes to homogenise and lyse the cells. The cells were then scraped and transferred into microtubes. One hundred microlitres of chloroform was added and the tube was vortexed vigorously for 15 seconds and then incubated at room temperature for 3 minutes. The tubes were spun at 12,000 RPM for 15 minutes at 4°C. This caused the separation of the aqueous and organic layers. The clear upper aqueous layer (approximately 500 µl) contained the RNA and to it an equal volume of isopropanol was added and left at -20°C overnight. The precipitate was centrifuged for 15 minutes at 14,000 RPM and 4^{o}C. The isopropanol was aspirated very carefully, and the pellet washed twice with 0.8mL of 75% ethanol by resuspending and centrifugation 5 minutes at 14,000 RPM and 4°C. After the last wash, the ethanol was removed carefully, and the pellet was air-dried on ice with cling film on top and the 1.5 mL tube lid open for 10 minutes, small holes were punched in cling film to allow air exchange. The RNA was resuspended with 45µl RNase free water and incubated at room temperature for 10 minutes, vortexed and incubated on ice for 10 minutes before measuring concentrations using NanoDrop. The RNA was stored in -80^{o}C.

To the lower organic layer after separation containing DNA and protein 0.3 mL of 100% ethanol was added. Samples were mixed by repeated inversion and incubated at room temperature for 2-3 minutes. The DNA was pelleted by centrifugation at 2,000g for 5 minutes at 4°C and the phenol-ethanol supernatant transferred to a new tube. The supernatant was used for protein extraction where 750µl of isopropanol was added to the phenol-ethanol supernatant and incubated for 10 minutes and centrifuged for 10 minutes at 12,000RPM at 4°C to pellet the proteins. The supernatant was aspirated and discarded with a micropipette. The protein pellet was resuspended with 1mL of 0.3 M guanidine hydrochloride in 95% ethanol to precipitate the proteins by incubation for 20 minutes. The suspension was centrifuged for 5 minutes at 10,000 RPM at 4°C and supernatant discarded with a micropipette. This step was repeated twice. 2mL of 100% ethanol was added and mixed by vortexing briefly. The tubes were incubated for 20 minutes before being centrifuged for 5 minutes at 10,000RPM at 4°C and the supernatant aspirated and discarded with a micropipette. The protein pellet was air dried for 5–10 minutes and the pellet resuspended in 200 µL of 1% SDS by pipetting up and down. To ensure complete resuspension of the pellet it was incubated at 50°C heat block. The quality was checked using NanoDrop and stored at -20^{o}C.

The pellet containing the DNA after organic layer centrifugation was resuspended in 500µl of 0.1M sodium citrate in 10% ethanol with pH of 8.5 and incubated for 30 minutes, mixing occasionally by gentle inversion. The sample was centrifuged then for 5 minutes at 2000 × g at 4°C and the supernatant was aspirated and discarded. The pellet was washed again and then resuspended in 750µl of 75% ethanol, before incubation for 10–20 minutes, mixing occasionally by gentle inversion. The sample was centrifuge for 5 minutes at 2000 × g at 4°C and the supernatant was aspirated and discarded as described above. The DNA pellet was air dried for 5–10 minutes and resuspended in 300µl of 8mM NaOH by pipetting up and down. The sample was centrifuged for 10 minutes at 12,000 × g at 4°C to remove insoluble materials and the supernatant transferred to a new tube. The pH was adjusted to 7.5 with HEPES buffer and 1 mM EDTA was added. The DNA was stored at -20^{o}C.

Preparation of cDNA

The High-Capacity cDNA Reverse Transcription kit was used. RT kit components were thawed on ice. The volumes required of each reagent were calculated according to the number of reactions needed. The master mix was prepared and kept on ice as described in (Table-8). Ten microlitres of the RT master mix was added to each reaction tube, followed by the addition of 10µl of RNA sample. The tubes were sealed and centrifuged to eliminate air bubbles. The reaction tubes were kept on ice until loading on the thermal cycler. The thermal cycler was programmed as 25˚C for 10 minutes, 37˚C for 120 minutes, 85˚C for 5 minutes and the reaction was then held at 4˚C. The reaction volume was set to 20µl, the reaction tubes were loaded, and the run started. Upon completion the cDNA was stored in a fridge at 2-6 ˚C for short term storage or in the freezer -15 to -25 ˚C for longer terms.

Reagent	Volume in µL
10X RT Buffer	2.0
25X dNTP Mix (100mM)	0.8
10X RT Ramdom Primers	2.0
MultiScribe™ Reverse Transcriptase	1.0
Nuclease Free Water	4.2
Total per Reaction	10.0

(Table-8) Preparation of Master mix for cDNA synthesis using High-Capacity cDNA Reverse Transcription kit.

Using a master mix reduces pipetting errors and disparities between samples.

Myoblasts mitochondrial membrane potential

Myoblasts seeding density optimisation

Human skeletal Muscle Myoblasts (HSMM) from a 35-year-old Caucasian female donor with BMI of 31 were resurrected and expanded to passage 2 using Basal media with the addition of SkGM SingleQuots supplement pack consisting of human Epidermal Growth Factor (hEGF), Fetuib, Bovine Serum Albumin (BSA), Dexamethasone, Insulin, and Gentamicin/Amphotericin-B (GA).

Comparison between the use of cover slips and 4-well chamber slides

Myoblasts were seeded on cover slips and into 4-well chamber slides for comparison. Four seeding densities were tested on 4-chamber slide; these were 5000, 10,000, 15,000 & 20,000 cells per chamber. Cells were also seeded on coverslips to compare visualisation under confocal microscopy.

Media comparison

Two T75 flasks were seeded with the same seeding density of myoblasts. One flask was grown in DMEM while the other flask was grown in the manufacturer recommended media; SKGM. Growth was monitored daily under light microscopy and media was changed daily for both flasks. After 8 days the experiment was terminated.

HSMM mitochondrial membrane potential (MMP) measurements

Measurement of myoblast mitochondrial membrane potential was conducted using $5x10^3$ cells per coverslip. Cell were treated with 20HE at ranging concentrations (0 ,1, 5 & 10 µM). After 24 hours of treatment, cells were washed with HEPES buffered physiological salt solution three times and then incubated in 30 nM TMRE for 30 minutes to stain the mitochondria. After the incubation, TMRE fluorescence was excited and measured at >590nm using a Leica confocal microscope. Further these parameters were measured following uncoupling with FCCP.

2.2.2.3 Molecular mechanisms investigation

Proteome analysis

Frozen liver tissue from uPA +/+ SCID mice were ground using pestle and mortar in liquid nitrogen and then homogenised using Dounce all glass tissue grinder in RIPA buffer to extract proteins. Samples were then centrifuged for 20 minutes at 14000RPM and 4°C. The supernatants were collected and stored in three aliquots at -80°C until further analysis. Calorimetric detection and quantification of total protein for all samples was estimated using Pierce BCA protein assay kit according to the manufacturer's instructions. Albumin (BSA) Standards were diluted and prepared and the microplate protocol was used. Twenty-five microliters of standards and samples were loaded on a U-bottom plate and 200µl of the kit working reagent was added to each standard and sample. This was then covered and mixed by vortexing for 15 seconds. The plate was then incubated at 37°C for 39 minutes before it was cooled to room temperature and finally the absorbance was measured at 562nm on Tecan Plate Reader.

Protein gel run and fragmentation

Following protein concentration estimation, protein samples of chimeric mice were diluted to 0.85mg/mL. From each sample 20µL were mixed with 4µL of 6X reducing buffer to prepare for electrophoresis on 15% ExcelGel with running conditions of 600V, 50mA and 30W for 45 minutes. The gel was then washed with 40% Ethanol, 10%Acetic acid wash solution. Each sample well was then excised to 8 fragments corresponding to the protein ladder.

In-gel protein digestion

All 8 protein fragments of all 10 samples were placed individually in a 96well plate. In-gel protein digestion using Trypsin/Lys-C Mix, Mass Spec Grade V5072 was conducted according to manufacturer's instructions. Gel pieces were washed by incubation in 200µL of 25mM ammonium bicarbonate at room temperature for 15 minutes followed by removing the supernatant and additional incubation in 200µl of acetonitrile at room temperature for 15 minutes. These alternating steps of incubation were repeated three times for a total of four washes. Reduction/Alkylation of the gel pieces was done by adding 200µl of 10mM DTT in 25mM ammonium bicarbonate and incubation at 65°C for 30 minutes. The supernatant was removed and the above washing steps in ammonium bicarbonate and acetonitrile were repeated four times. Then, 200µL of freshly prepared 10mM iodoacetamide in 25mM ammonium bicarbonate was added and the plate was incubated in the dark for 45 minutes at 37°C. The wash steps were then repeated four times. The gel pieces were then digested with minimal volume (20µL) of 20ng/µL Trypsin/Lys-C Mix solution for 10 minutes. The gel pieces were then covered in 60µL of 25mM of ammonium bicarbonate and incubated overnight at 37°C. Ten microliters of 1% formic acid was added and the supernatants were collected for analysis. Proteome analysis was conducted by LC-MS/MS using the Orbitrap ELITE.

Transcriptome analysis

RNA and protein extraction from Liver

Liver tissue from all samples were weighed and divided into two halves for RNA & protein extractions. RNA extraction was done as explained in the previous experiment by grinding the frozen tissue in liquid nitrogen. TRIzol was added to disrupt the cells and RNA was precipitated using alcohol precipitation method. Quality check was performed on the Bioanalyzer and Nanodrop. RNA samples were stored in -80°C until further analyses.

RNA sample preparation

RNA samples from SCID mice liver tissue treated with 20HE and placebo were prepared in triplicates for whole Transcriptome expression analysis using GeneChip WT PLUS Reagent Kit according to manufacturer's instructions. The three-day amplification and labelling process began with first and second strand cDNA synthesis reactions respectively from 100ng input. This was followed by an overnight *in-vitro* transcription for the synthesis and amplification of cRNA. cRNA is then purified using magnetic beads and quantified using the NanoDrop. Fifteen Micrograms of the purified cRNA is then used as input for the synthesis of 2nd cycle ss-cDNA (single stranded complimentary DNA) followed by RNA hydrolysis and removal by RNase H enzyme. The ss-cDNA was then purified and quantified using the NanoDrop and five micrograms were used as input for the fragmentation and the terminal labelling reactions. Finally, the labelled fragments of the ss-cDNA were hybridised to the array plate according to manufacturer's instructions for the GeneTitan instrument where the staining and the washing processes were automated.

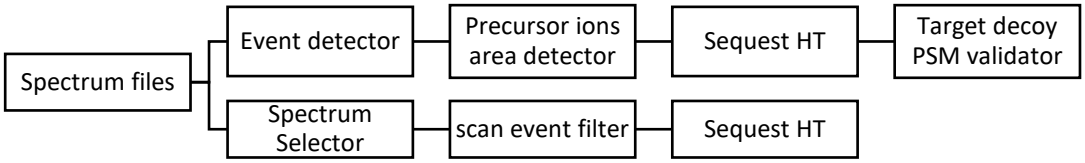
Data presentation and statistical tests

Data were analysed using Excel. Data are shown as mean (±SD), or for non-normally distributed data as median (interquartile range), in text and in tables. Comparison of treatments was by paired t-tests and the null hypothesis rejected at $p<0.05$.

Analytical methods

Proteomics data analysis

Raw Meat software and Proteome Discoverer PD were used for data analysis. Quantitative analysis method was created on Proteome Discoverer (PD)(Figure-13) to create (.msf) file type in which all the fractions per sample have been combined into one file. Individual sample (.msf) files were later loaded/opened together in the PD for comparison and easer analysis, i.e. one result page for chimeric-20HE treated group created from 4 (.msf) files of four samples. Peptide high confidence filtering (Xcorr cross correlation score) of 1.8, 2.4 and 2.5 for single, double and triple charge proteins respectively was used while loading the data in order to avoid false protein ID identification. The combined data per group, with high confidence peptides, are then exported to into a single excel file with a list of all proteins and peptides detected according to the set criteria high confidence. In excel, all keratin proteins were excluded which came from skin/hair contamination. Proteins that were not detected in all samples within one group were excluded. These will have 0 or no value in the coverage column (Column labelled: Score A, Score B, Score C and Score D for all 4 samples per group). All proteins that did not have a Score in at least one sample were also excluded. Proteins detected in both 20HE treated group and placebo treated group were excluded for the comparative proteomics but used for the quantitative proteomics. Each protein detected only in the 20HE treated group is then compared to the original (.msf) file of the placebo treated and if it was detected in at least one sample this protein was then excluded. This was done also for the proteins detected only in the placebo treated group. Proteins identified with only 1 unique peptide were excluded. The minimum of 2 unique peptides was required. Scans from raw files were viewed using RawMeat V2 where full scans represent parent peptides and MSMS scans show more peaks within the parent compound detected. Trypsin or lysin digestion controls were studied for all samples. Protein lists extracted from excel were further analysed using STRING which is an online data analysis tool that identifies protein-protein interactions based on the input protein list (91).



(Figure-13) Method Created on Proteome Discoverer (PD). PD is a tool on which a workflow is created -like the one created in this figure- to process raw data retrieved from the mass spectrometry system and compares them to databases of choice to identify the proteins detected.

Transcriptome data analysis

Data analysis was done using freely available software; Transcriptome Analysis Console TAC version 4.0 . Expression Analysis Settings used were fold change < -2 or > 2 and *p*-value < 0.05 was considered significant at gene level analysis. Expression was reported if ≥ 50% samples have detection above background (DABG) values below the Threshold. Exported gene lists were analysed using Microsoft Excel. Functional enrichment analysis was also conducted using TAC 4.0.

Combined functional enrichment analysis was conducted from data generated from the proteome and transcriptome analyses using g.profiler (92) which is a web-based analysis tool that shows possible enrichment of pathways based on the input gene lists. Protein lists were converted to gene names using UniProtKB.org (93) which allowed further analysis was conducted using STRING and FunRich software; a freely available for download software that conducts functional enrichment analysis.

Human *in-vivo* studies data analysis

data were entered and analysed using SPSS version 25 for Windows. The effect of the supplements was measured by the changes in the various parameters before and after the intervention in the same individual.

2.2.2.4 Validation

To validate NOS3 finding, mRNA and protein expression analyses were performed on human coronary artery endothelial cells (HCAECs) after treatment with various doses of 20HE.

Cell culture

HCAECs were thawed in a 37˚C water bath and seeded into a T75 flask pre-treated with attachment factor for 30minutes at 37˚C in Endothelial cells growth media. Media was changed every 72 hours. At 90% confluence cells were then trypsinised after three washes with PBS. After 1-minute incubation in trypsin, media was added, and cells were placed in a 15 mL tube and centrifuged at 220g for 5 minutes. Media was discarded, and the cell pellet was resuspended in 2mL of media. Cells were then seeded in 6-well plate that was pre-treated with attachment factor for 30 minutes at 37˚C. After reaching confluence, cells were starved for 24 hours using endothelial cells basal media to reduce effects of supplements on the cell growth. To prepare a 10mM stock solution of 20HE, 10mg of 20HE was reconstituted in 2mL of ethanol. Cells in 6-well plate were treated with 0.1nM, 1nM, 10nM, 100nM, 1µM and ethanol (control).

Protein extraction

After 24 hours, protein was extracted by removing the media from each well, washing twice with 1mL PBS and adding RIPA buffer. After scraping the cells off, the buffer containing the proteins was pipetted into a 15mL tube and centrifuged at max speed for 5 minutes. The protein supernatant was collected and used for NOS3 protein expression investigation by western blot using anti-eNOS antibody (Abcam).

Western blotting

Protein concentration estimation was conducted using BCA assay according to manufacturer's recommendations detailed in previous section. 20µg of total proteins were added to the reducing agent in LDS buffer and incubated at 70 ˚C for 10 minutes. The proteins were then loaded into SDS-PAGE gel, and the gel was run for 1 hour at 180V in MOPS running buffer. Proteins were the transferred to nitrocellulose membrane at 30V for 2 hours in transfer buffer (100mL methanol,25mL Transfer buffer topped to 500mL with dH_2O). Membrane was then blocked with 5% milk in TBST for 1 hour. The membrane was then incubated in anti-eNOS antibody (1 in 500) overnight. Membrane was then washed 3 times with PBST followed by incubation with secondary antibody (1 in 2000) for 1 hour and the membrane was washed 3 times. The membrane was then incubated in 2mL chemiluminescence and warped in cling film. In a dark room the membrane was exposed to

a film for 15 seconds then the film was washed in a developer then water then exposed to a fixer and finally washed with water.

RNA extraction

RNA was extracted by removing the media from each well, washing each well twice with 1 mL PBS and adding 350 µL lysis buffer. After scraping the cells on ice, the solution was placed in a 1.2mL tube and an equal volume (350µL) of ethanol was added. RNA was extracted using the Qiagen RNeasy kit according to manufacturer's recommendations. The 700µL solution was placed in the spin column provided by the kit and centrifuged at 13000RPM for 15 seconds. The flow through was discarded and the column was washed with 700µL RWI buffer and centrifuged for 15 seconds. The flow through was discarded. 700µL of buffer RPE was then added to the column and centrifuged for 15 seconds. Buffer RPE was added again and centrifuged for 2 minutes and the flow through was discarded. The column membrane was dried by centrifugation for 1 minute in a new collection tube. Finally, the RNA was eluted in 30µL of RNase free water.

qPCR Protocol

The RNA was used for NOS3 mRNA expression investigation by qPCR using Quantifast SYBR green RT-PCR kit where two master mixes were prepared for the extracted RNA to use with eNOS primer and the housekeeping gene (HKG) GAPDH for normalisation. To each 10µL of master mix, 2µL of cDNA ,converted from RNA, was added. The mixture was then loaded on the RT-PCR Thermal cycler.

CHAPTER 3

RESULTS. METABOLISM OF ECDYSTEROIDS

Ecdysteroids and specifically 20HE gained the attention of many recreational and elite athletes due to their reported anabolic effect (54,66,76). To investigate their effects in humans, and due to *in-vivo* human studies challenges, the previously described uPA+/+ SCID mouse model was used. This was done after confirming the model's ability to generate the human metabolites of Methandienone; gold standard anabolic doping agent which was tested after single and multiple dosing experiments. Gene expression studies using drug metabolism arrays were carried out using extracted RNA from liver tissue of the experimental mice. Further, human excretion study was also conducted in one volunteer *in-vivo* using a 20HE containing supplement presenting an opportunity for extended investigation of 20HE and its effects in humans *in-vivo* using 20HE containing supplement described in the following chapter.

3.1 Metabolism of Methandienone

3.1.1 Urinary metabolites of Methandienone

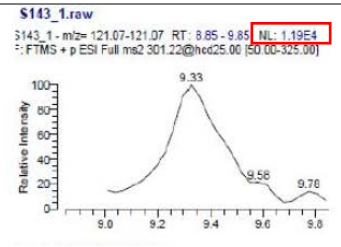
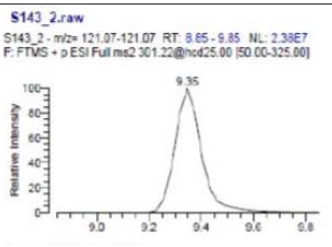
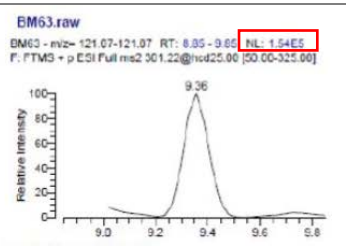
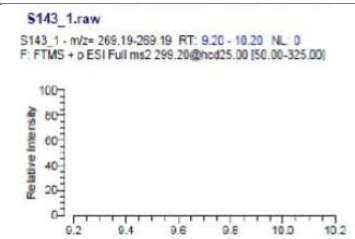
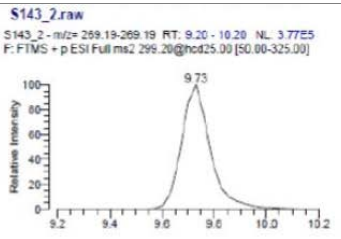
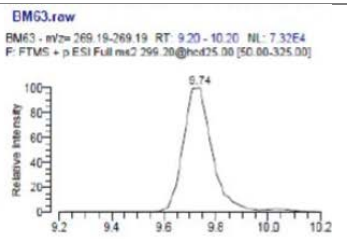
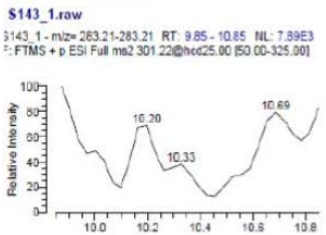
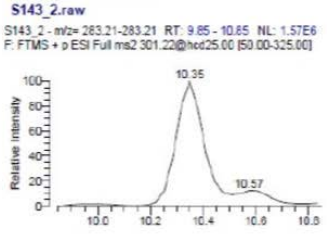
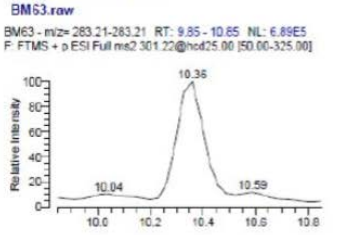
Human hepatic metabolism of Methandienone was assessed using liquid and gas chromatography (LC & GC) coupled with mass spectrometry. Liquid chromatography analyses require the compounds of interest to be soluble in the mobile phase used in the process. Whereas, gas chromatography analyses require the compounds of interest to be derivatized with a mixture of MSTFA/NH4I/2-propanethiol to have better volatility and is therefore more suited for smaller compounds. LC, on the other hand, is considered more suitable for both small and very large molecules.

The parent compound Methandienone, the metabolite 17-Epimethandienone and the long-term metabolite 18-nor-17beta-hydroxymethyl,17alpha-methyl-androst-1,4,13-trien-3-one were analysed Using LCMS (Table-9). The other metabolites were analysed using GCMS including Epimtendiol, 6bOHmethandienone and 5beta-methyltestosterone (Table-9).

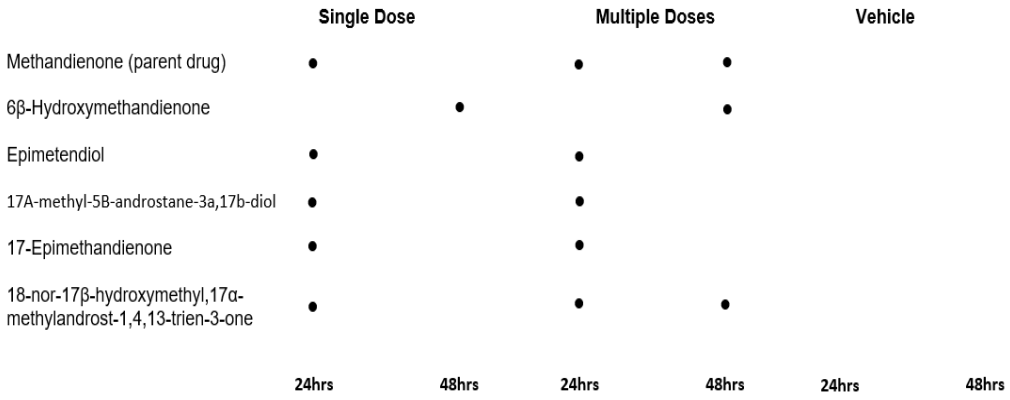
LCMS analysis of urine showed the presence of previously reported metabolites of Methandienone, including the long-term metabolite, in the drug-treated cohort but not in placebo treated cohort (Figure-14). Methandienone was detected 24 hours after drug administration in both single and multiple doses treated cohorts. After multiple dosing Methandienone remained detectable up to 48 hours after last administration indicating increased detectability in response to multiple dosing which was in line with what's been reported in the literature (76). This was also observed with the long-term metabolite 18-nor-17beta-hydroxymethyl,17alpha-methyl-androst-1,4,13-trien-3-one (Table-10).

Analyte	Analysis type	Detection
17-epimethandienone	LC	✓
20B-OH-NorMD	GC	✓
Methandienone	LC	✓
6b-OH-metandienone	GC	✓
18-nor-17beta-hydroxymethyl,17alpha-methyl-androst-1,4,13-trien-3-one	LC	x
5beta-methyltestosterone	GC	✓
18,17b-dihydroxy-17a-methylandrosta-1,4-dien-3-one	GC	✓
16a,17b-dihydroxy-17a-methylandrosta-1,4-dien-3-one	GC	✓
NorEMD(18-normetenol)	GC	x
Epimetendiol	GC	x
20A-OH-NorMD	GC	x

(Table-9) Methandienone and the detectability of its urinary metabolites using GCMS and LCMSMS analyses. Analyses were conducted using urine samples collected from the uPA +/+ SCID mice administered with methandienone where over 60% of analytes investigated were detected using the model.

	BLANK URINE	SIGLE-DOSE	POSITIVE CONTROL
Methandienone			
Long-term metabolite			
17-Epimethan--dienone			

(Figure-14) LCMS chromatograms of urine samples before (BLANK URINE) and after single dose administration of methandienone (Left and middle columns respectively). The x-axis shows relative intensity of the peak detected plotted against retention time on the y-axis. The right column shows results from the positive control. The parent compound Methandienone was detected 24 hours after single administration of the drug and in the positive control. The peak shown in the blank urine is considered background because of its low relative intensity indicted by the circled Normalization Level NL=1.19E4 compared to the positive control with NL=1.54E5. The long-term metabolite of methandienone; 18-nor-17beta-hydroxymethyl,17alpha-methyl-androst-1,4,13-trien-3-one and 17-epimethandienone were detected 24 hours after administration and in the positive control (right column). No peak was detected in the blank urine.



(Table-10) LCMSMS and GCMS analyses of urinary metabolites of Methandienone from uPA +/+ SCID mice. Mice were administered with either a single dose or multiple doses of 20HE and urine samples were analysed by LCMS and GCMS. Table shows that the parent drug and its metabolites were detected 24 hours after the first administration of the drug in single and multiple doses. One metabolite: 6β-Hydroxy Methandienone was only detected 48hours after first administration of the drug. Multiple doses increased the sensitivity of detection after 48hours. None of metabolites were detected in the placebo treated group.

Comparing results from this study to studies from literature where the first study was conducted on healthy human volunteer taking 25mg Methandienone and the urinary metabolites were analysed by GCMSMS (66) and the second study by Lootens et. al. (76) was conducted using the uPA+/+ SCID mouse model that was used in the current study administered with a similar concentration of Methandienone; 40µg per gavage. The metabolites that were assayed in this study compared to the other two studies are presented in Table-11. It was found that there were three of the metabolites detected in the human urinary study (66) but were not detected in studies utilising the uPA +/+ SCID mice which were conducted separately in this project and by Lootens et.al (76). Though it represents 27% of total assayed metabolites this might signify one of the limitations of this alternative model. Adding on to the urinary investigation, methandienone metabolism in the uPA +/+ SCID mice model was further investigated at the RNA expression level using mouse and human drug metabolism arrays detailed in the following section.

Analyte	Parr et. al. (66)	Lootens et.al. (76)	This study
EpiMD	✓	✓	✓
20B-OH-NorMD	✓	✓	✓
Metandienone	✓	✓	✓
6b-OH-metandienone	✓	✓	✓
NorMD	✓	✓	X
5B-MT	✓	X	✓
18-OH-MD	✓	**Not Assayed**	✓
16A-OH-MD	✓	**Not Assayed**	✓
NorEMD(18-normetenol)	✓	X	X
EMD(Epimetendiol)	✓	X	X
20A-OH-NorMD	✓	X	X

(Table-11) Comparison of Methandienone metabolites detected by three independent studies. The study conducted by Parr et al. (66) was carried out in one human subject given an oral dose (5mg) of the drug, the Lootens et al. (76) was a study carried in the same uPA +/+ SCID mouse model as the one used in this study. The multiple dosing from this study appeared able to detect more of the expected metabolites.

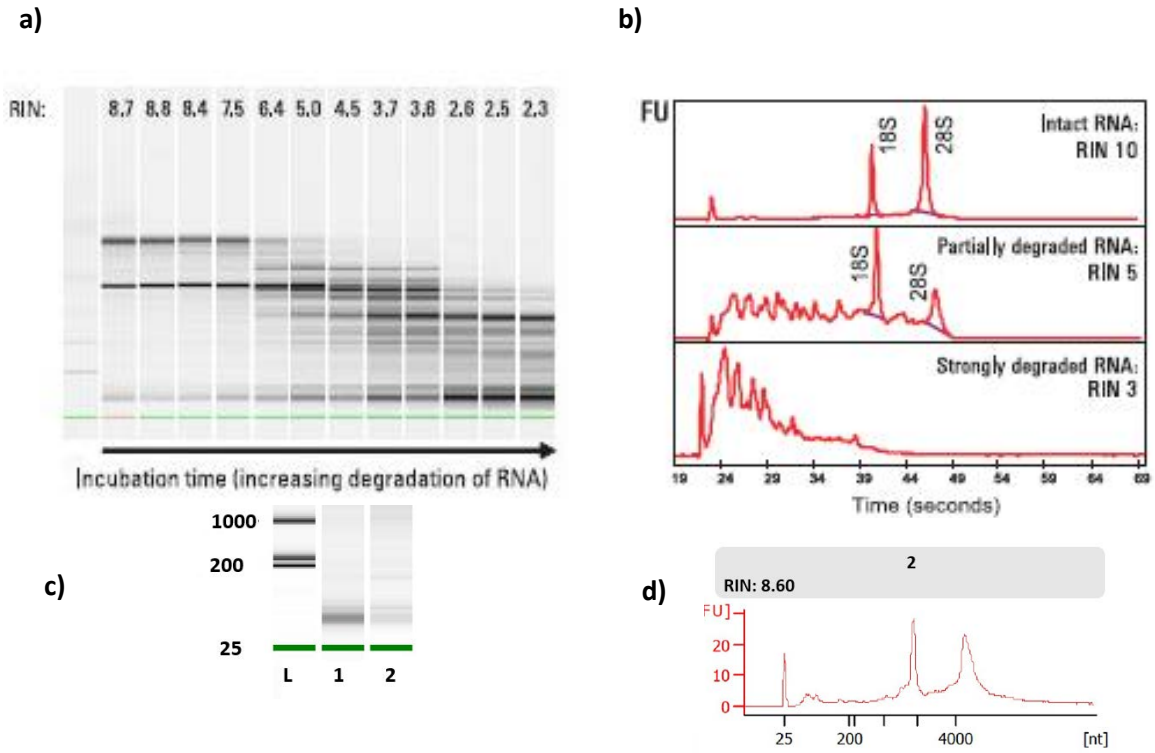
3.1.2 Profiler array analysis

RNA extraction

Human hepatic metabolism of Methandienone was also investigated at the level of gene expression using human phase I drug metabolism enzymes. RNA was extracted from the livers of all the animals, concentrations and quality check was conducted on the Nano Drop and Bioanalyzer. Some molecular analyses require specific nucleic acid quantity and quality and profiler array analysis requires the sample to be of specific integrity to ensure successful and reliable results. The nanodrop is an absorbance-based technique to assess RNA quantity is calculated based on absorbance at 260nm and quality is assessed by estimating the presence of protein and other contaminants based on the measurement at 280 and 230 nm respectively (86). The main disadvantage in using the Nanodrop is the lack of sensitivity as it does not distinguish between nucleic acids while on the other hand the Bioanalyzer is specific to the sample of interest (RNA). The bioanalyzer is an electrophoresis-based instrument where the nucleic acid is combined with fluorescent dye. The software presents the results as electropherograms that include the size and quantity (86) (Figure-15). It provides each RNA sample with an integrity number (RIN) based on an algorithm at a scale of 1 to 10 where 10 is the highest RNA integrity (86). All the RNA samples used for the profiler array analysis had to have an RNA Integrity Numbers (RIN) of greater than 5 (Figure-15). Results indicated that RNA concentrations were too high and thus all samples were diluted, and quality check was repeated. In addition, quality PCR (qPCR) was conducted on all extracted RNA samples after conversion to cDNA using human β-actin the results are presented in the (Table-12).

		NanoDrop	Bioanalyzer		RTPCR
Sample ID	Group code	RNA Conc. (ng/µl)10Xdilution	RNA Conc. (ng/µl) 10Xdilution	RIN	β-actin Ct
S143	SCD	734.9	211	6.8	32.093
S140	SCD	937.2	129	7.1	20.424
S035	SCD	419.8	17	7.5	19.859
S034	SCV	628	20	6.0	20.237
R909	SCV	1215	70	2.2	20.438
S036	SCV	618.6	240	6.6	19.225
R711	SND	797.2	281	6.7	31.688
R700	SND	1211.4	194	8.1	33.355
R701	SNV	3141.7	157	2.9	33.406
R704	SNV	1768.9	181	5.7	33.645
S053	MCD	491.1	30	2.6	20.847
S356	MCD	1080.8	183	2.3	22.179
S049	MCD	883.5	70	2.4	22.426
S050	MCV	1027.6	247	7.1	20.173
S355	MCV	946.7	124	7.5	21.001
S052	MCV	996.9	141	N/A	22.443
S460	MND	1687.3	179	6.2	31.983
M-A218	MND	1936.4	103	6.1	33.771
M-A216	MNV	492.1	99	7.3	19.428
S458	MNV	926.2	1052	6.8	30.809

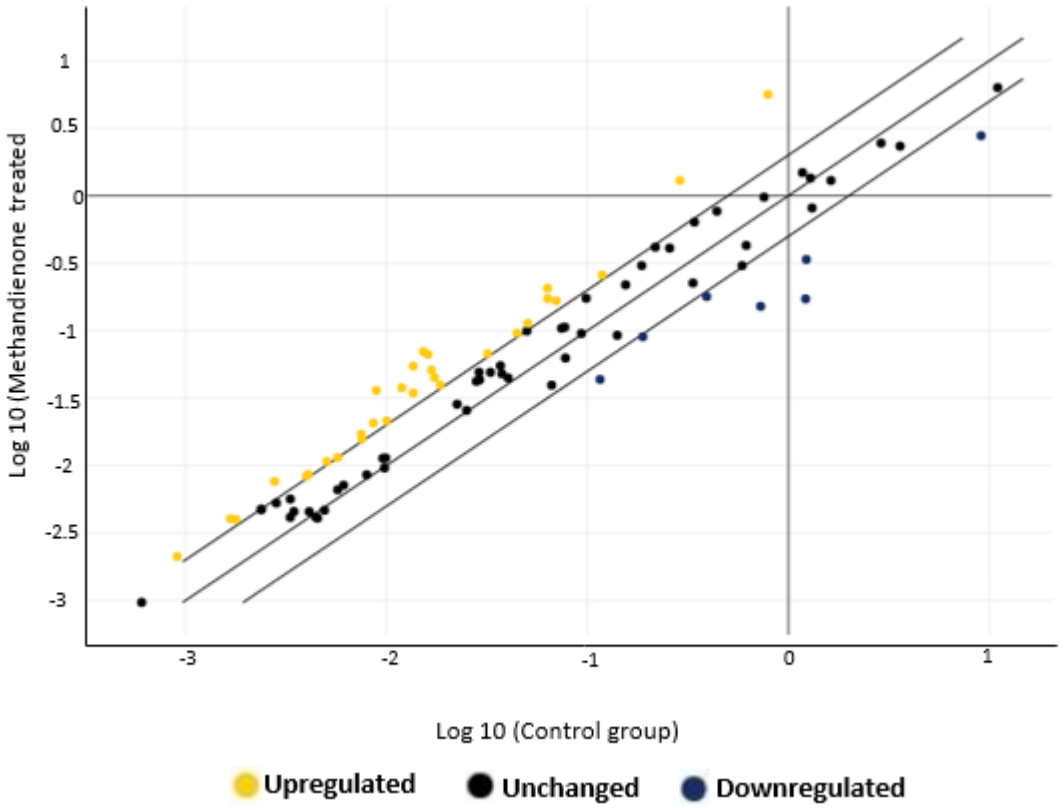
(Table-12) Analyses of RNA extracted from uPA+/+ SCID mouse livers following treatment with Methandienone. RNA samples with a RIN score/value of greater than or equal to 5 were deemed acceptable. The group code refers to S: Single Dose, M: Multiple Doses, C: Chimeric, N: Non-Chimeric, D: Drug treated, V: Placebo treated).



(Figure-15) Quality and degradation check of extracted RNA using Agilent Bioanalyzer. Panel (a) shows degree of RNA degradation in the gel and this is related to the densitometric analysis and RIN shown in (b). These figures are from the manufacturer for reference. (c) represents comparison between my own data sample 1 with RIN of 3.50 and Sample 2 with RIN of 8.60. (d) the chromatogram indicating the quality of the RNA where in sample 2 (bottom) RIN:8.50 clearly showing two distinct peaks of 18S and 28S whereas sample 1 the peaks are weak and merged.

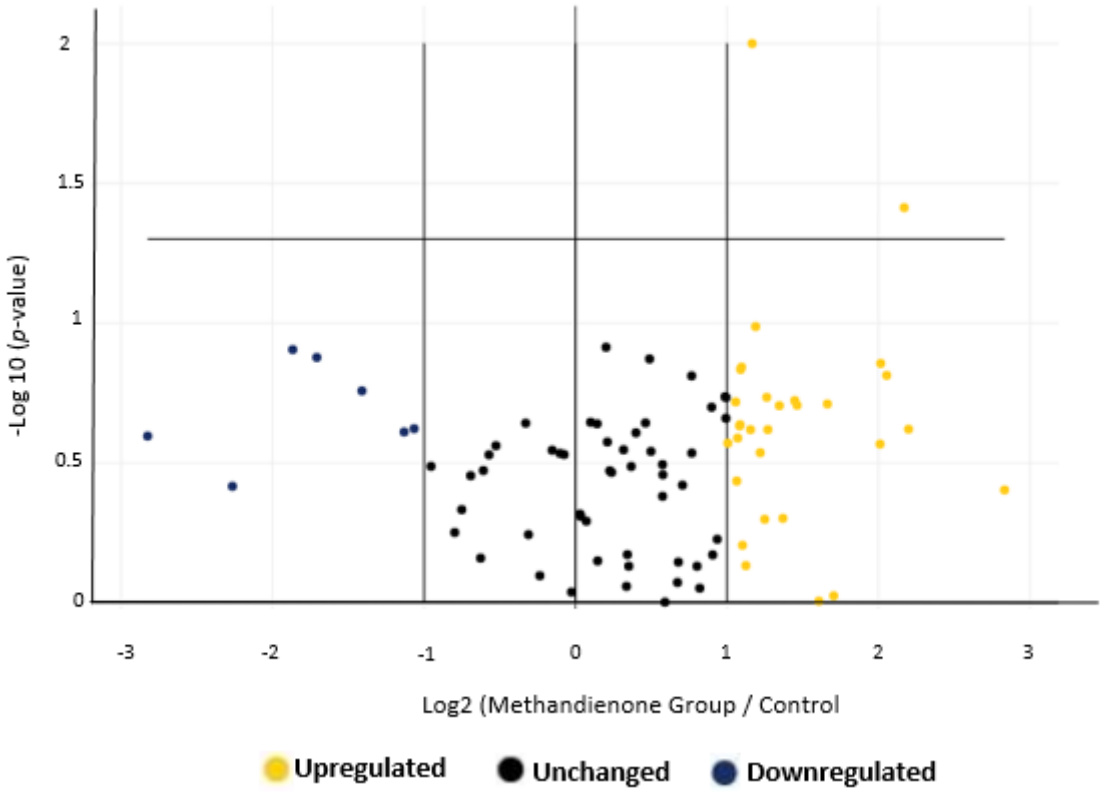
Mouse Phase I enzymes drug metabolism array

Mouse phase I drug metabolism enzymes profiler arrays were used to examine the response of the humanised liver of the chimeric mice to mouse drug metabolism genes and to compare the responses to that of the non-chimeric mice. Synthesized cDNA from RNA extracted from the SCID mice livers were used for analysis on the mouse phase I enzymes drug metabolism array. Fold change differences in gene expression of the chimeric mice either treated with methandienone or placebo is presented in a scatter plot (Figure-16).

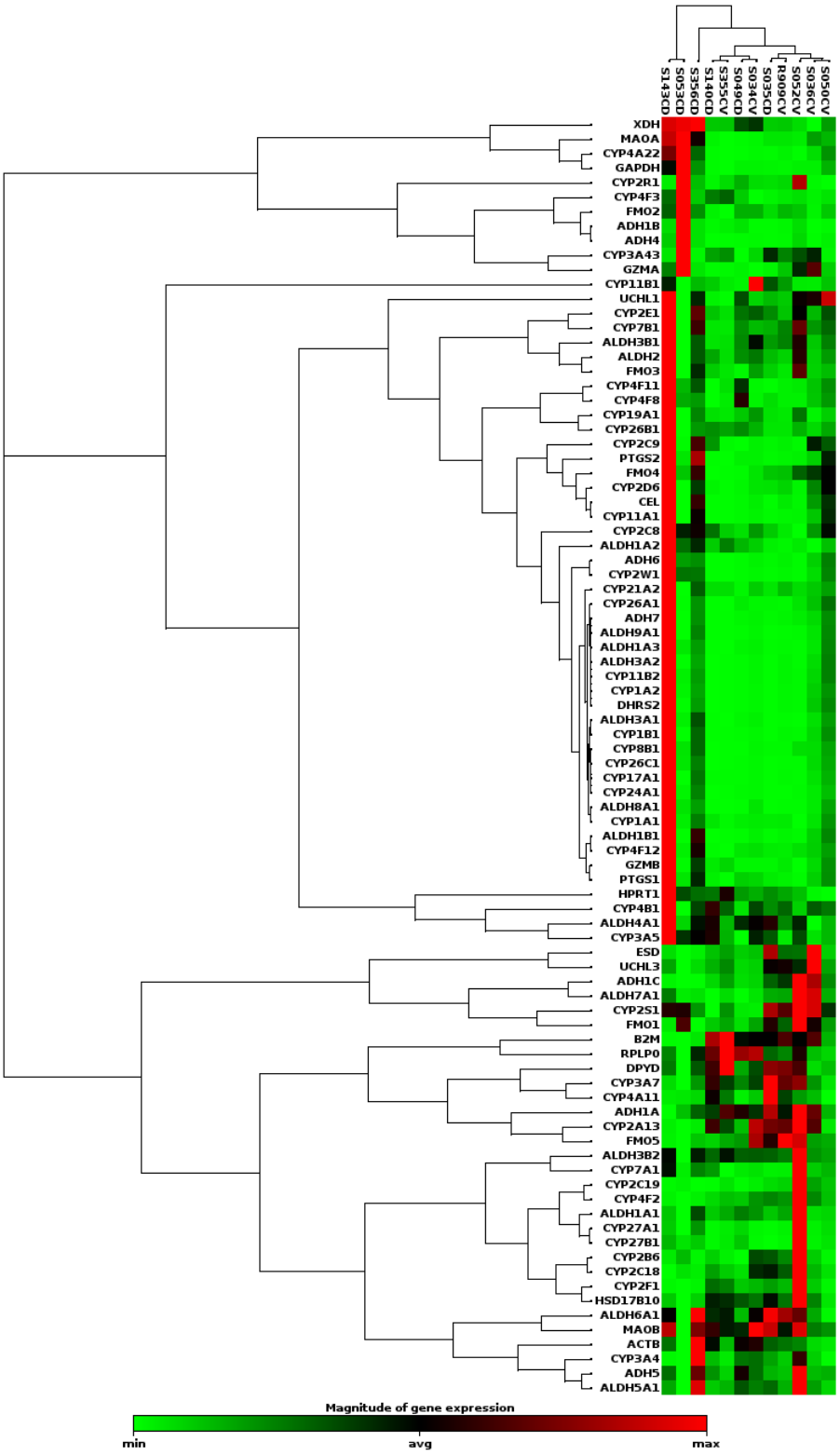


(Figure-16) Scatter plot presenting the expressed genes in the drug treated group after normalisation against the control group. Genes that did not show changes in expression after treatment with the drug are indicated by the central line and the dotted line represent the fold change cut-off (2.0). Genes that met the fold change threshold are indicated in yellow (upregulated) in blue (downregulated).

Significantly differentially expressed genes with *p*-value≤0.05 were plotted against fold change in a volcano plot (Figure-17) and non-hierarchical clustering is illustrated in (Figure-18). It was observed that significantly upregulated genes were more than the downregulated genes. These included CYP1A2, CYP27A1 and ALDH3A2 and genes significantly downregulated were FMO1, ADH5 and CYP2C54.

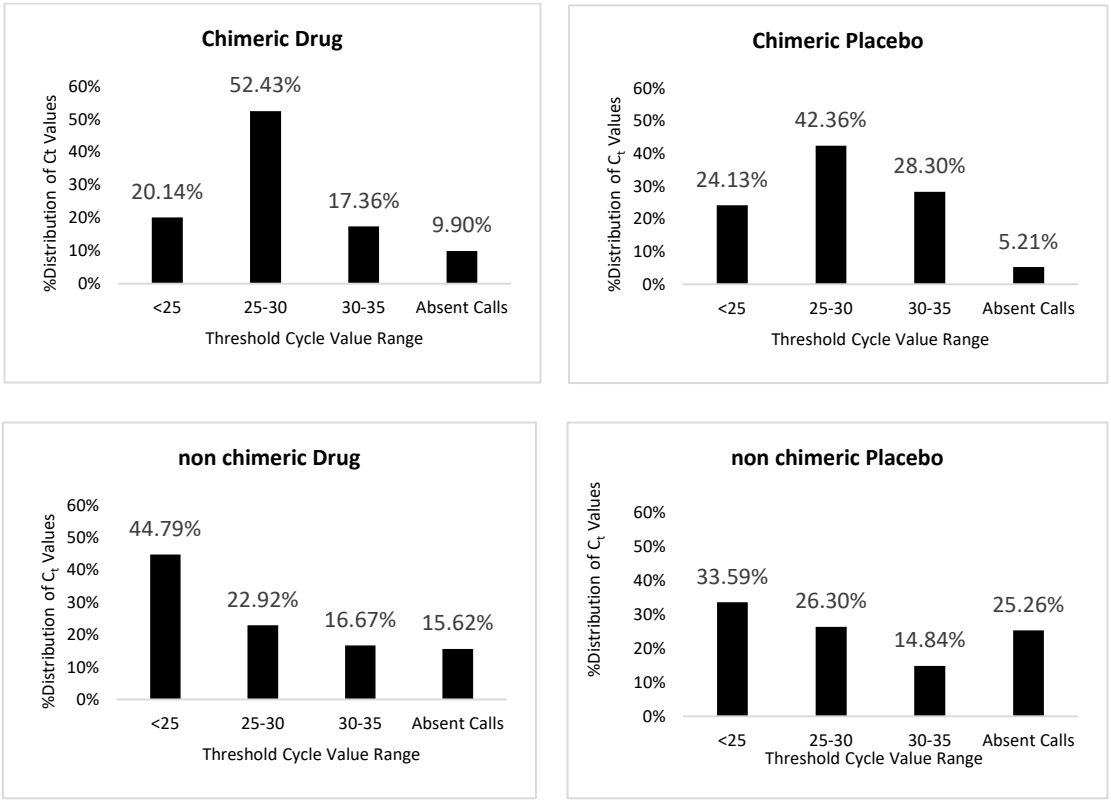


(Figure-17) Volcano plot showing genes that were significantly differentially expressed (*p*-value ≤0.05) (y-axis) plotted against fold change (x-axis). Significantly upregulated genes are indicated in yellow and significantly downregulated genes indicated in blue.



(Figure-18) Two-dimensional non-supervised hierarchical clustering of both methandienone and control group gene expression. Heat map and dendograms indicating co-regulated genes at a sample or group level are displayed.

As the mouse phase I drug metabolism enzymes profiler array analysis was conducted on both chimeric/non-chimeric and drug/placebo treated cohorts, it was observed that the non-chimeric mice mounted little response to the drug since the uPA +/+ SCID mice suffer from transgene-induced liver disease that renders the mouse liver non-functional. (Figure-19). While lower Cycle threshold (Ct) represents over expression, it was observed that the Ct distribution for the chimeric mice was higher than that observed in the non-chimeric mice after treatment with either placebo or drug. Comparing Chimeric and non chimeric grups both drug treated, cycle value range in the non chimeric was mostly less than 25 while the chimeric group had higher Ct in comparison since it is mouse array.



(Figure-19) Percent Distribution of Values in mouse Phase I drug metabolism array. Samples from groups of mice given either single or multiple doses were combined to increase study power. Lower cycle threshold values indicate higher expression. Threshold cut off was set at >35.

Human Phase I enzymes drug metabolism array

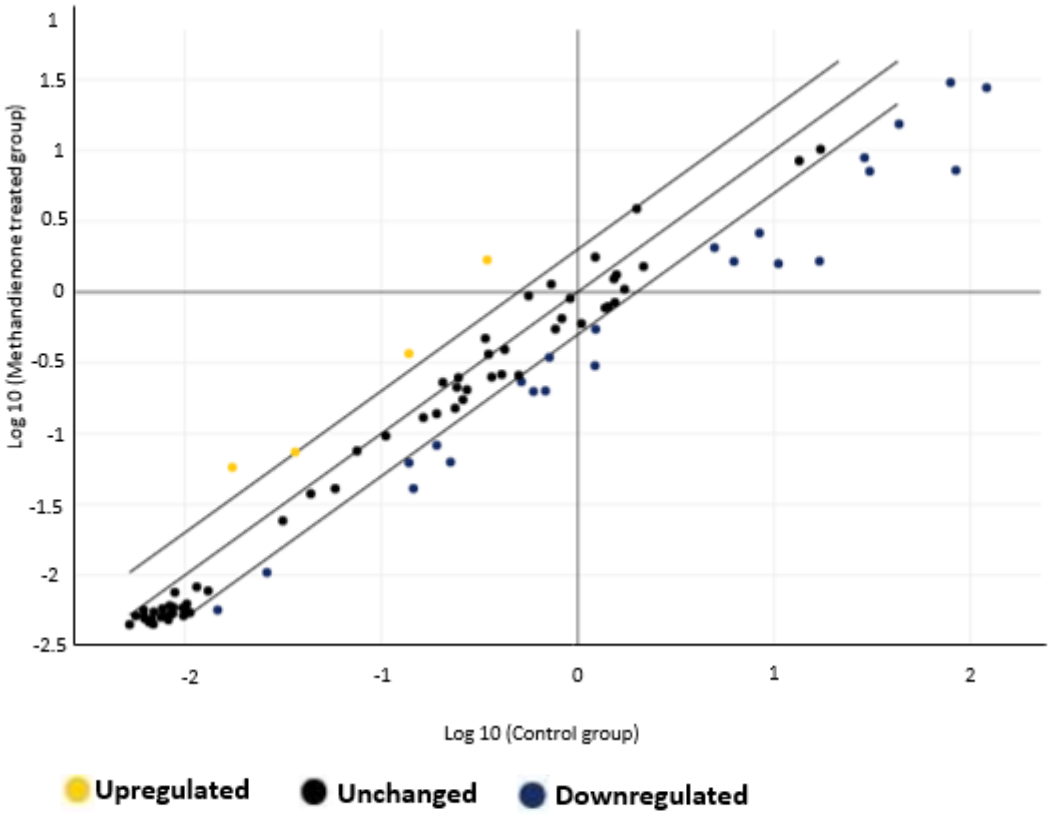
Human phase I drug metabolism enzymes expression was investigated using profiler arrays (Qiagen). The same cDNA that was reverse transcribed from the SCID mice livers was also used to investigate the expression of the genes expressing drug metabolising enzymes that are involved in the Human phase 1 drug metabolism processes. Prior to the experimental data analyses, RNA and PCR quality assurance was carried out using the online SA Biosciences software (Table-13).

In the chimeric animals that had been successfully transplanted with human hepatocytes i.e those measured with significantly elevated systemic human albumin, there was a global up-regulation of the Phase 1 metabolism enzymes in response to methandienone. Data analysis, using ß-actin, B2M and HPRT1 as the house-keeping genes for normalisation and excluding samples that did not pass the QC checks, showed the mRNA expression of the following genes was significantly induced by the drug, compared to placebo: ALDH7A (1.33 fold difference, $p< 0.001$), ALDH9A1 (2.26 fold difference, $p=0.04$) and ALDH6A1 (1.88 fold difference, $p=0.08$).Figure-20 shows the fold difference between the two groups and the significance is presented in the volcano plot (Figure-21). Cluster gram of both groups is also presented in (Figure-22). Adding on to this finding, significance was also observed in the expression of genes from the aldehyde dehydrogenases (ALDH) superfamily of enzymes, an ALDH activity assay was conducted using plasma obtained from the SCID mice.

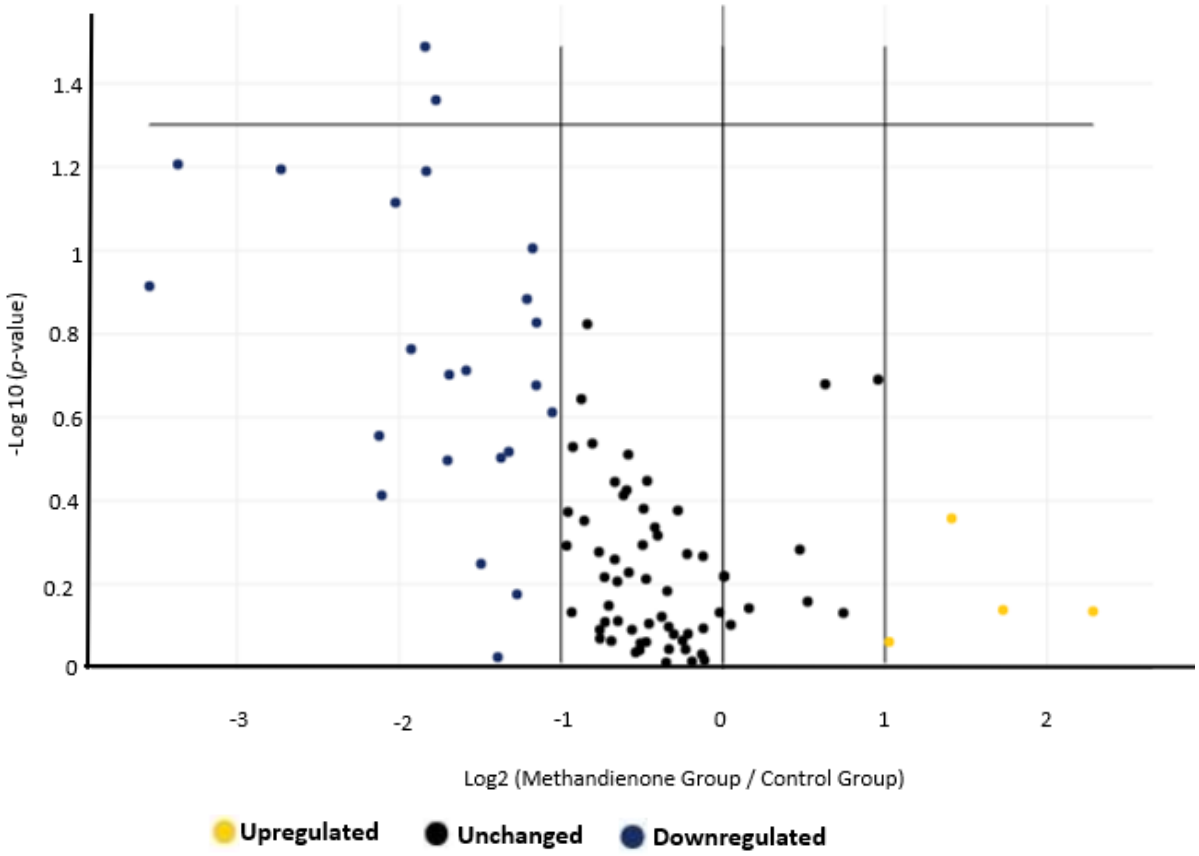
65% percent of the genes in the pathway specific array were induced after drug treatment as opposed to 52% following placebo, this was particularly apparent when looking at genes that showed Ct of between 25-30 (Figure-23A). The non-chimeric mice did not mount a xenobiotic response to the drug, probably because their residual liver function was compromised (Figure-23B).

Chimeric mice single dose						
	Methandienone treated			Placebo treated		
Samples	35	140	143	34	36	909
PCR array reproducibility	Pass	Pass	Pass	Pass	Pass	Pass
RT efficiency	Pass	Inquiry	Pass	Pass	Inquiry	Pass
Genomic DNA contamination	Pass	Pass	Inquiry	Inquiry	Inquiry	Pass
Chimeric mice multiple dose						
	Methandienone treated			Placebo treated		
Samples	53	49	356	52	50	355
PCR array reproducibility	Pass	Inquiry	Pass	Inquiry	Pass	Pass
RT efficiency	Pass	Pass	Pass	Pass	Pass	Pass
Genomic DNA contamination	Pass	Pass	Pass	Pass	Pass	Pass

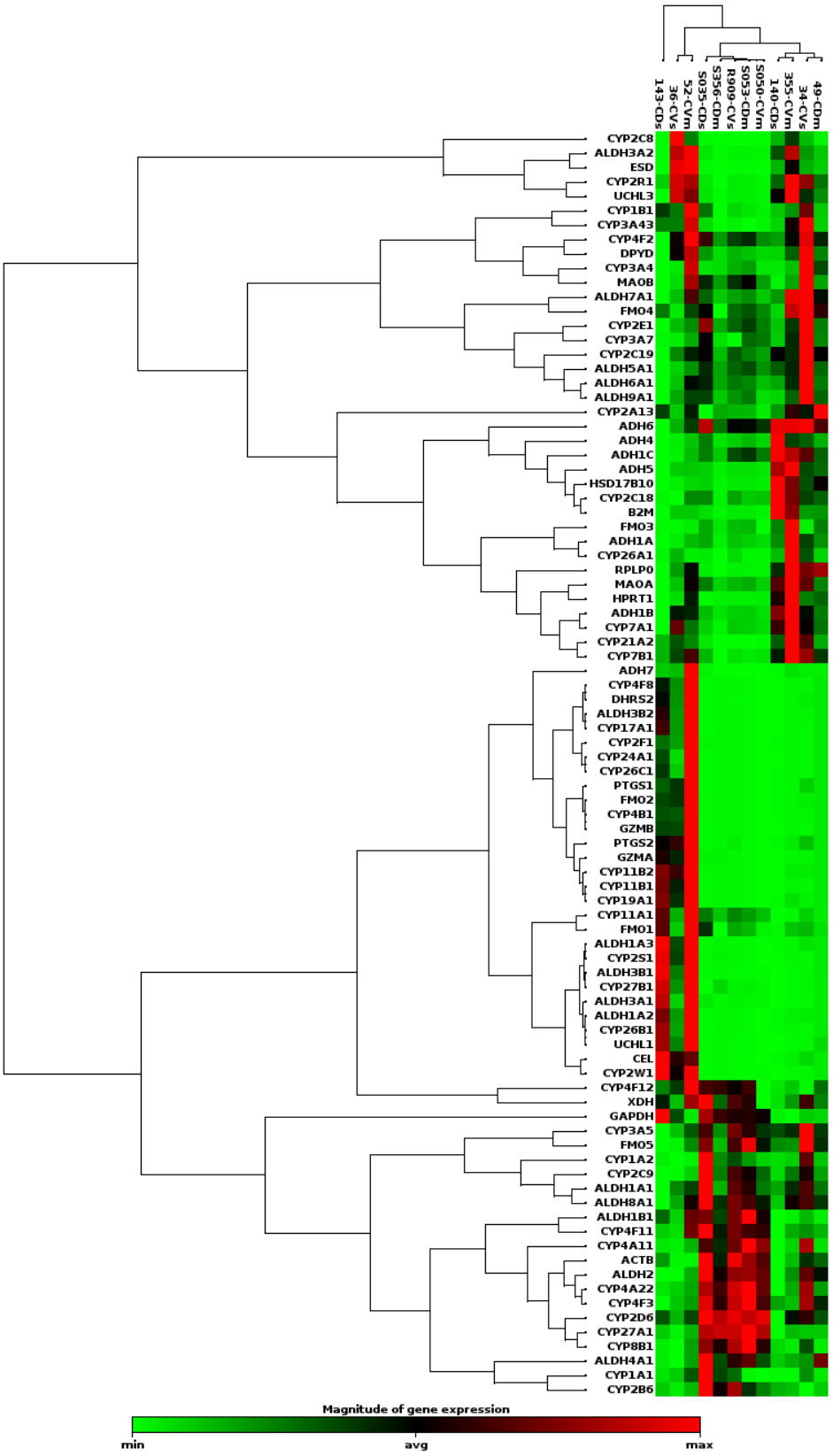
(Table-13) Quality Assurance check of Human Phase I drug metabolism array conducted using SA Biosciences online software. Samples in the top table were treated with a single dose of Methandienone and the bottom samples were treated with multiple doses. This quality check shows the reproducibility of the array, the reverse transcription efficiency testing the conversion of RNA to DNA and finally detection of DNA contamination.



(Figure-20) Scatter plot presenting normalised expressed genes after methandienone treatment against untreated group based on fold change. Central line shows unchanged expression and the upper and lower lines represent the fold change cut-off (2.0). Genes that met the fold change threshold are indicated in yellow (upregulated) in blue (downregulated).

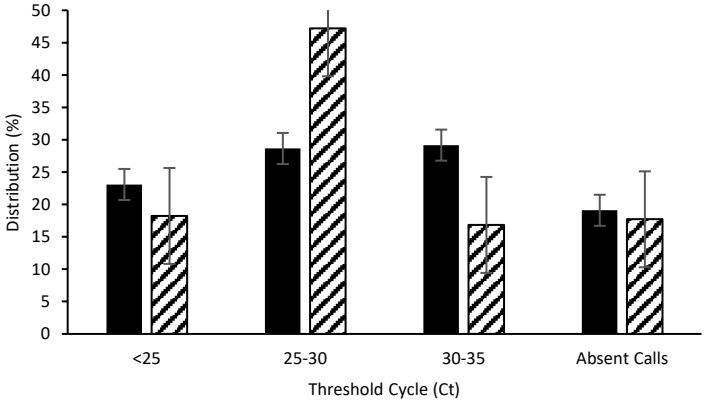


(Figure-21) Volcano plot showing significant changes in gene expression (*p*-value ≤0.05) plotted against fold change. Upregulated genes with positive fold change indicated in yellow dot. Blue dotts present significantly downregulated genes. Black dots indicate unchanged expression after drug treatment.

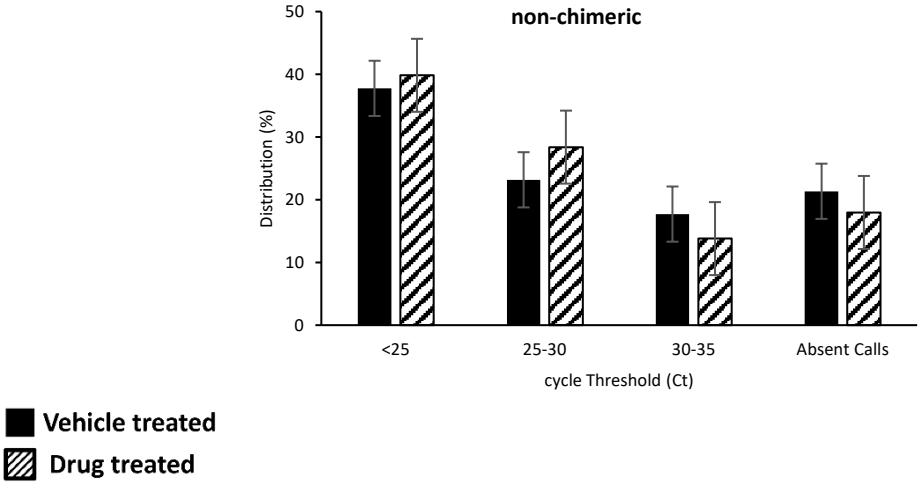


(Figure-22) Clustergram of methandienone and control group gene expression. The heat map and dendograms indicating co-regulated genes at a sample or group level are displayed.

a)



b)

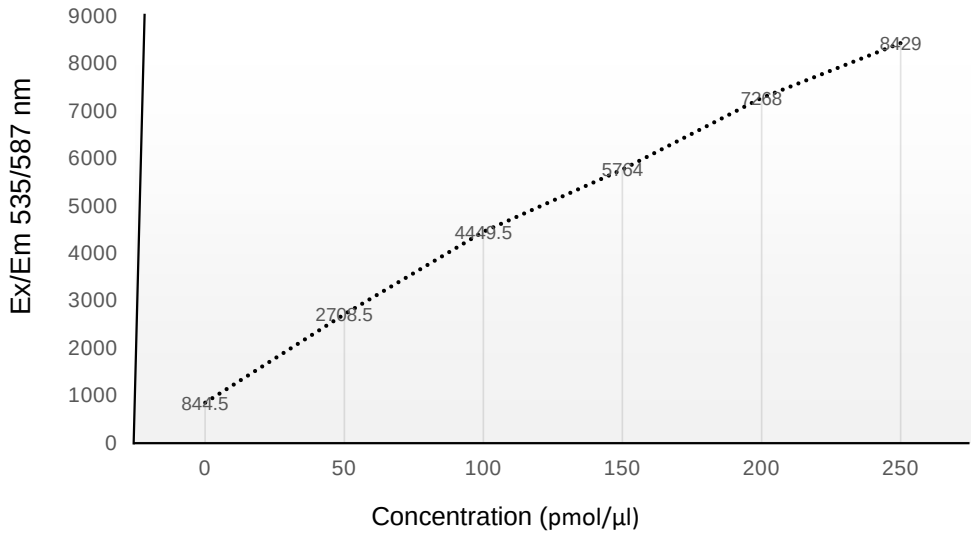


(Figure-23) Up-regulation of mRNA expression of phase I enzymes on human Phase I drug metabolism array. The mRNA expression of a significant number of the phase I enzymes were up regulated following injection with the drug, compared to placebo, in the liver of chimeric mice using a human array (a). No such drug effect was seen in the non-chimeric mouse liver using a mouse array (b), suggesting the lack of functionality of the organ in the host.

Aldehyde dehydrogenase activity in blood

An upregulation of aldehyde dehydrogenase enzymes (ALDH) was observed from the profiler array investigation. ALDH superfamily consists of 19 genes expressing key Phase1 cellular detoxification enzymes that oxidise various aldehydes and generate the corresponding carboxylic acid. There are three major classes of aldehyde dehydrogenases Class 1 and Class 3 are cytosolic and include both constitutive and induced forms and Class 2 is a constitutive mitochondrial form. The activity of these enzymes was tested on the plasma obtained from the chimeric SCID mice using a highly sensitive fluorescent kinetic assay where the limit of NADH sensitivity ranged between 1 to 5 pmol/µl. Acetaldehyde is oxidised by ALDH forming NADH that incorporates to the kit's PicoProbe generating potent fluorescence (Ex/Em = 535/587) with sensitive detection of as low as < 0.05 mU ALDH activity (Figure-24).

Despite the higher detection sensitivity of the kit used, levels of ALDH activity in the samples were extremely low, but detectable. While there was a slight increase in the plasma ALDH activity after multiple, compared to the single dosing, this did not reach significance (Single 1.32 (0.6) versus multiple 1.56 (0.5) pmol/µl; p=0.45).



(Figure-24) Calibration Curve for Aldehyde dehydrogenase activity assay. A slight increase in ALDH activity was observed in response to methandienone treatment. Increased ALDH activity levels was higher in the multiple doses group in comparison to single dose.

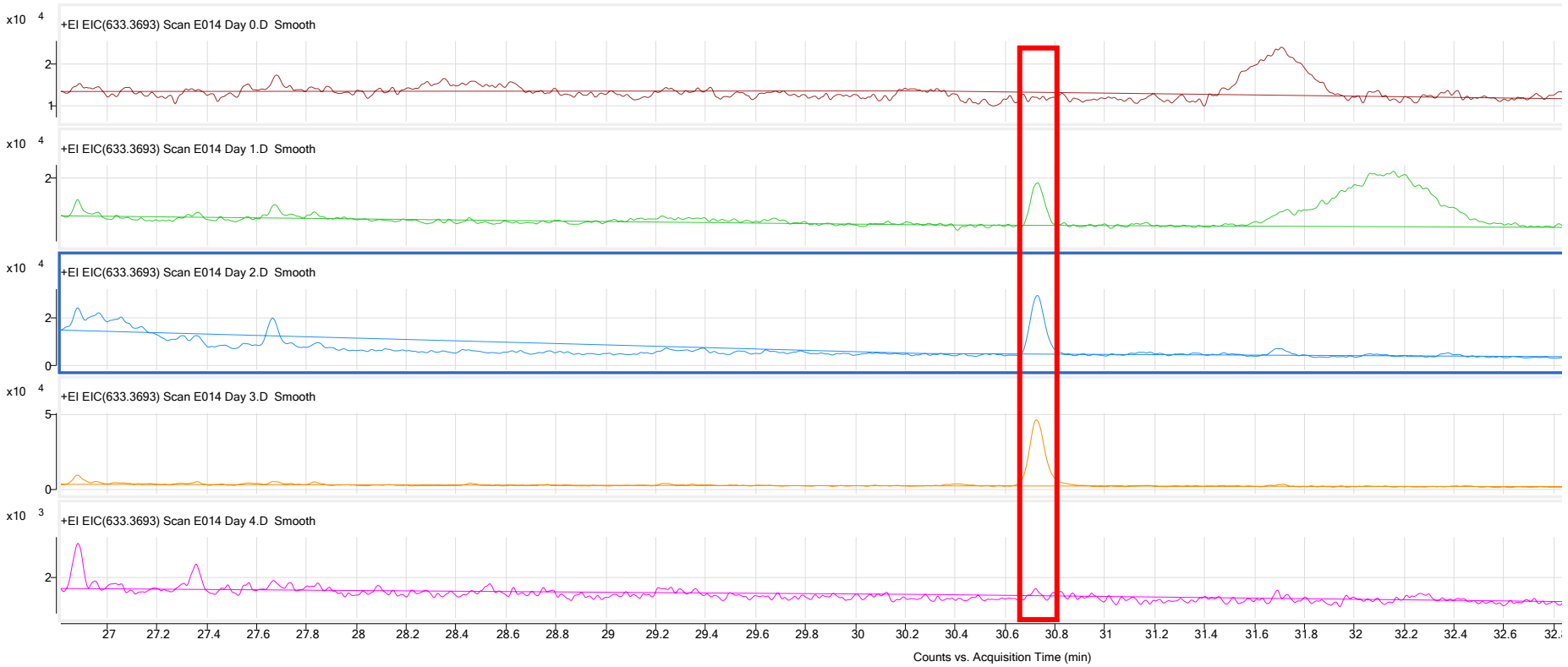
Results from this section have guided the studies that followed. First the uPA +/+ SCID mouse model and based on the urinary metabolite profiling of Methandienone proved to be suitable model for the investigation of human hepatic metabolism of any drug including ecdysteroids. Also, multiple dosing of the drug increased the sensitivity of detection which was applied in the experiment that followed; human hepatic metabolism of 20HE using the uPA +/+ SCID mice. Hepatic gene expression analysis provided specific insights on the effects of methandienone on the expression of 84 key drug metabolising enzymes and while methandienone is widely investigated in the literature this provided a good tool for the investigation of 20HE effects on hepatic drug metabolism gene expression profile.

3.2 Metabolism of 20HE

3.2.1 Urinary metabolites of 20HE

In a similar manner to the experiments conducted to investigate urinary metabolites of methandienone, urinary metabolite profiling was also conducted on urines collected from uPA +/+ SCID mice administered with multiple doses of 20HE or placebo by oral gavage. Results showed that the parent compound remained detectable up to 24 hours after administration in the 20HE treated group and by the end of the experiment on Day 4 the parent drug was not detected (Figure-25). Day 4 of the experiment represented 48 hours after the last 20HE administration. As expected, 20HE was not detected in the placebo treated group.

Since the data on 20HE metabolism is sparse and the human metabolites of 20HE reported in literature by Tsitsimpikou et al (69) and Brandt et al.(70) (Table-2) have not yet been validated, it was difficult to obtain standards of these metabolites in order to investigate their presence in the urinary profile. In addition, attempts in novel metabolites detection in response to 20HE is being further pursued but outside the scope of this project.



(Figure-25) Results from gas chromatography from chimeric mouse urine samples before and after multiple-dose administration of 20HE. Top row represents blank urine prior to the administration of the drug. The following three rows of chromatograms show the parent compound 20HE detectable 24 hours after administration. The bottom row presents the undetectability of 20HE 48 hours after last administration of the drug.

3.2.2 Profiler array analysis

RNA extraction from Liver

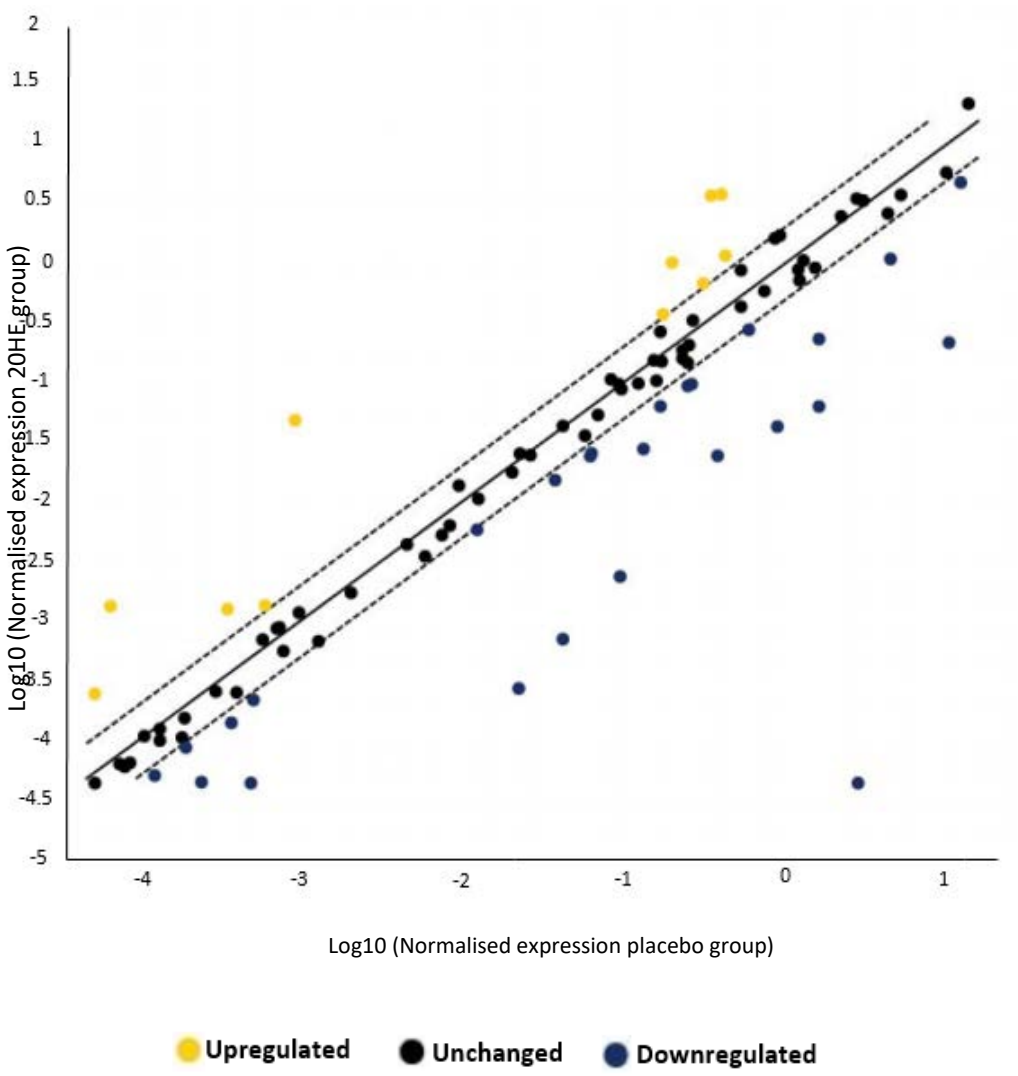
To investigate the hepatic gene expression profile of the drug metabolising enzymes in response to 20HE, a pathways specific gene expression profiler array was used. This investigation can help in identifying the possible reactions and modifications from phase I & II metabolising enzymes conducted to the parent compound by the humanised liver. Livers harvested from the animal were used for this investigation. Liver tissues were weighed and divided for RNA and protein extraction. Liver weights ranged from 0.4-0.7 g halved for RNA and protein extractions. This amount proved to be very high giving samples that are high in quantity and quality. All samples were diluted for downstream analyses. Results from RNA extraction are summarised in (Table-14). The extracted RNA samples were then and reversed transcribed to cDNA and were loaded on the arrays.

Sample	Group code	Conc. (ng\µl)	260/280	260/230	Conc.(ng\µl) 1:100 Dilution	260/280	260/230
5	CV	7654.6	1.95	1.16	997.9	2.04	1.18
9	CV	5124.9	2.04	1.26	1119.7	2.04	1.09
16	CV	4887	1.95	0.65	1889.1	1.97	0.66
18	CV	3997.5	1.89	0.56	1124.7	1.96	0.51
7	CD	7488.7	1.92	0.84	908.9	1.96	0.73
12	CD	7556.2	1.96	0.93	1119.1	2.02	0.87
14	CD	6010	1.98	0.79	789.9	2	0.64
29	CD	6952.9	2.03	1.39	1762	2.02	1.39
19	NV	6600.9	1.97	1.24	1194.5	2	1.19
966	ND	6753.4	1.99	1.1	1075.9	2.03	0.98

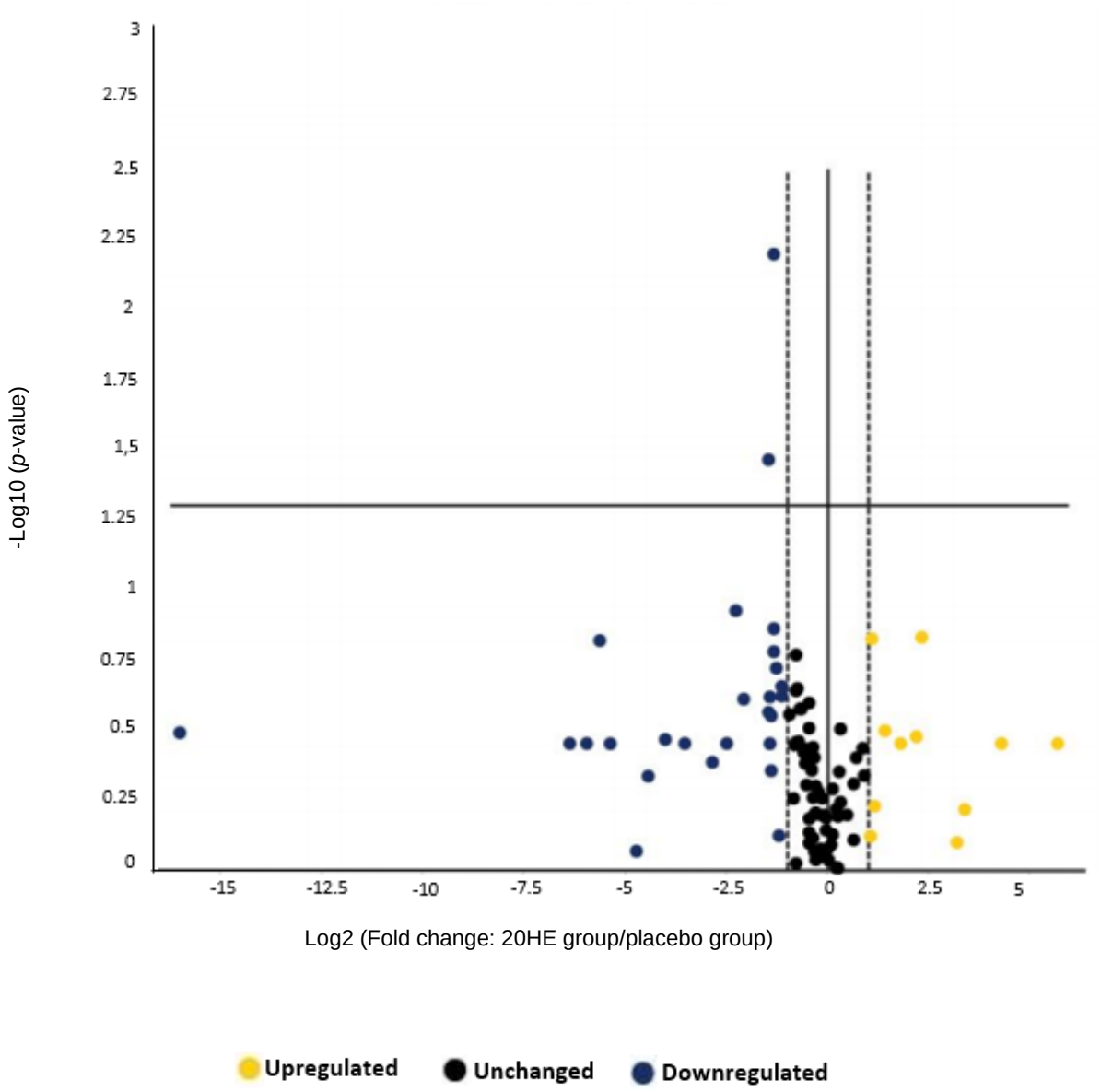
(Table-14) NanoDrop RNA concentrations extracted from the harvested livers of mice treated with either drug of placebo. Quality ratios are also indicated where RNA is considered pure if the 260/280 ratio is ≈ 2.0 and 260/230 ratio < 2 indicates presence of contaminants. These measurements were repeated after 1:100 dilution. Group code refers to C: chimeric, N: non-chimeric, V: placebo treated and D: drug treated.

Human drug metabolism array

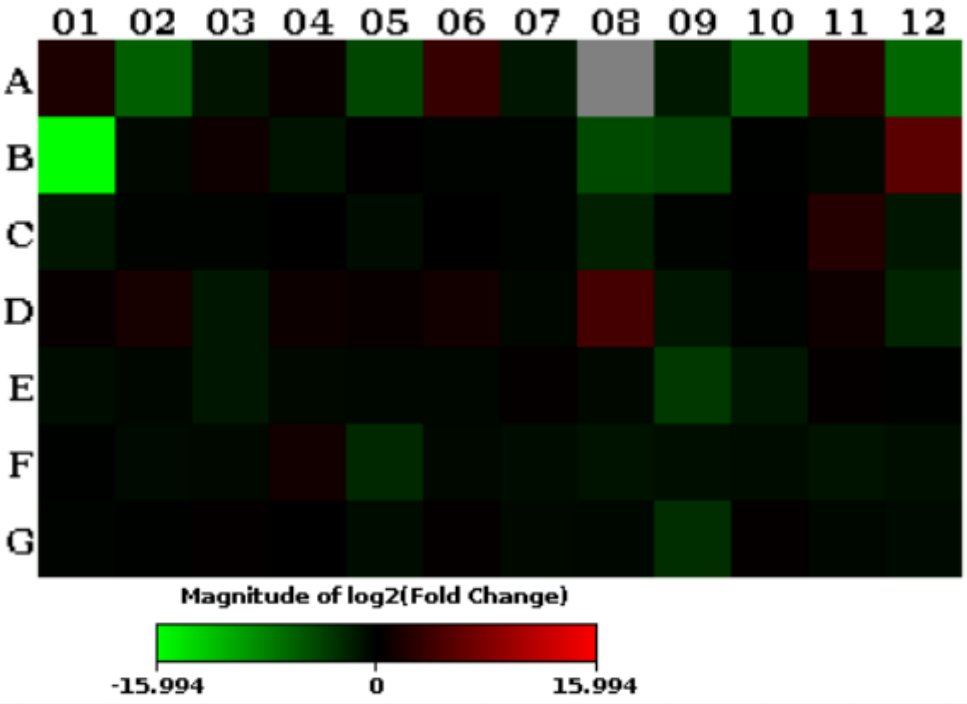
As previously described PCR quality assurance was carried out using the online SA Biosciences software and results indicated that 15 genes were overexpressed in the 20HE treated group compared to placebo group and 29 were under expressed (Figure-26 and 27). As expected, upregulation in the Cytochrome P450 enzymes including CYP11B2, CYP17A1, CYP19A1, CYP2E1 and CYP2F1 was observed. CYP19A1 (Cytochrome P450 family 19 sub-family A member 1) is a gene that encodes the protein (enzyme) aromatase which is involved in the conversion of androgen hormones to oestrogen (Figure-28) (87). Another interesting Cytochrome P450 family of enzymes member that was also differentially expressed in response to 20HE in the drug metabolism array was CYP17A1 which plays an important role in the steroid biosynthesis, the clustering of the genes and the fold changes are shown in Figures 29 and 30. Nitric Oxide Synthase3 (NOS3) was also upregulated in the 20HE treated group. NOS3 is a protein coding gene involved in several processes and most importantly it is involved in the relaxation of smooth muscle cells vasculature (Figure-30).



(Figure-26) Scatter plot of normalised expressed genes of the groups administered with 20HE and placebo plotted against each other. Unchanged genes are indicated in the central line surrounded by the dotted line representing the fold change cut-off (2.0). The upregulated (yellow) and downregulated (dark blue) present genes that met the fold change threshold of 2.0.

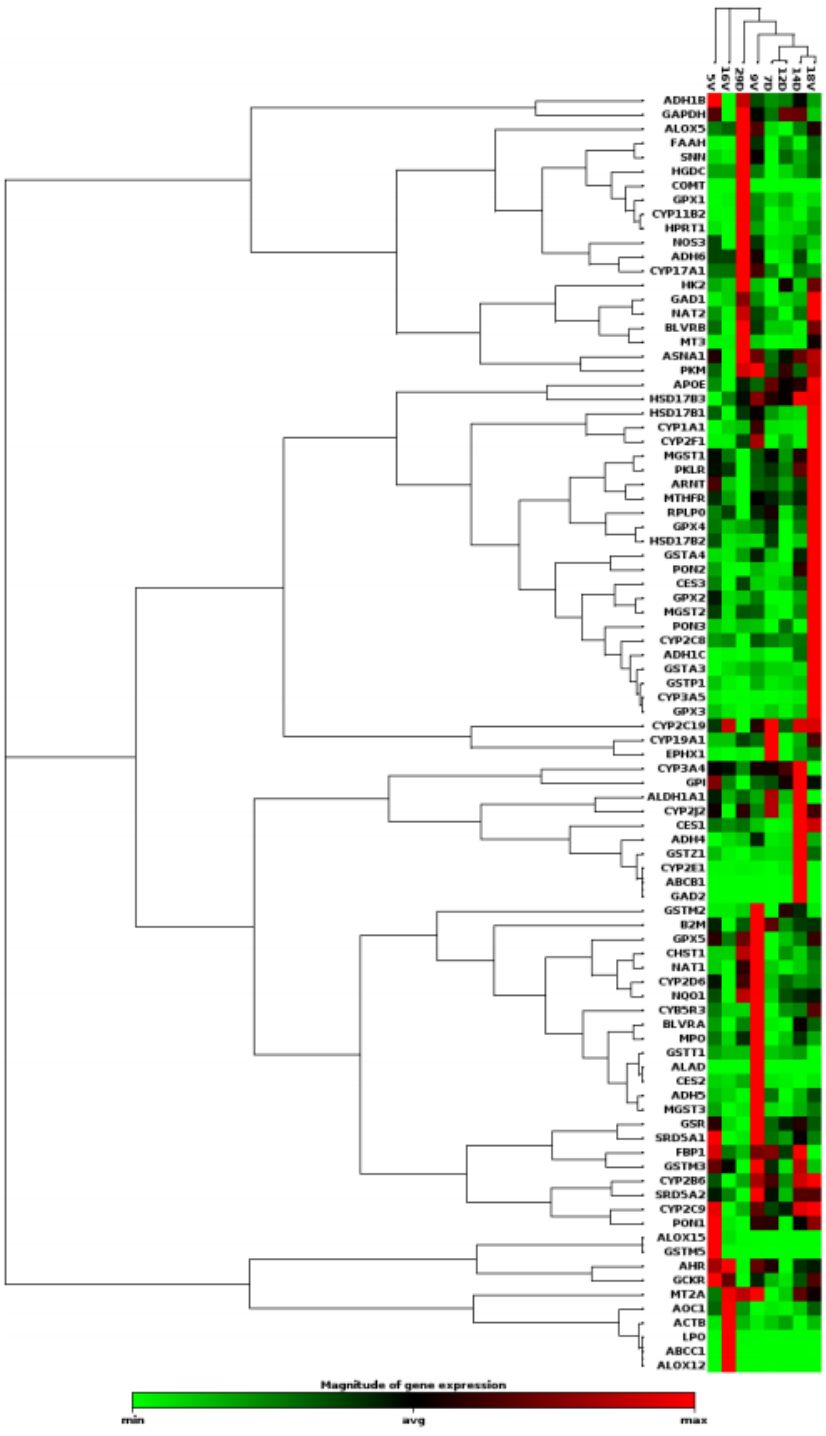


(Figure-27) Volcano plot presenting significantly differentially expressed genes (*p*-value ≤ 0.05) plotted against fold change.

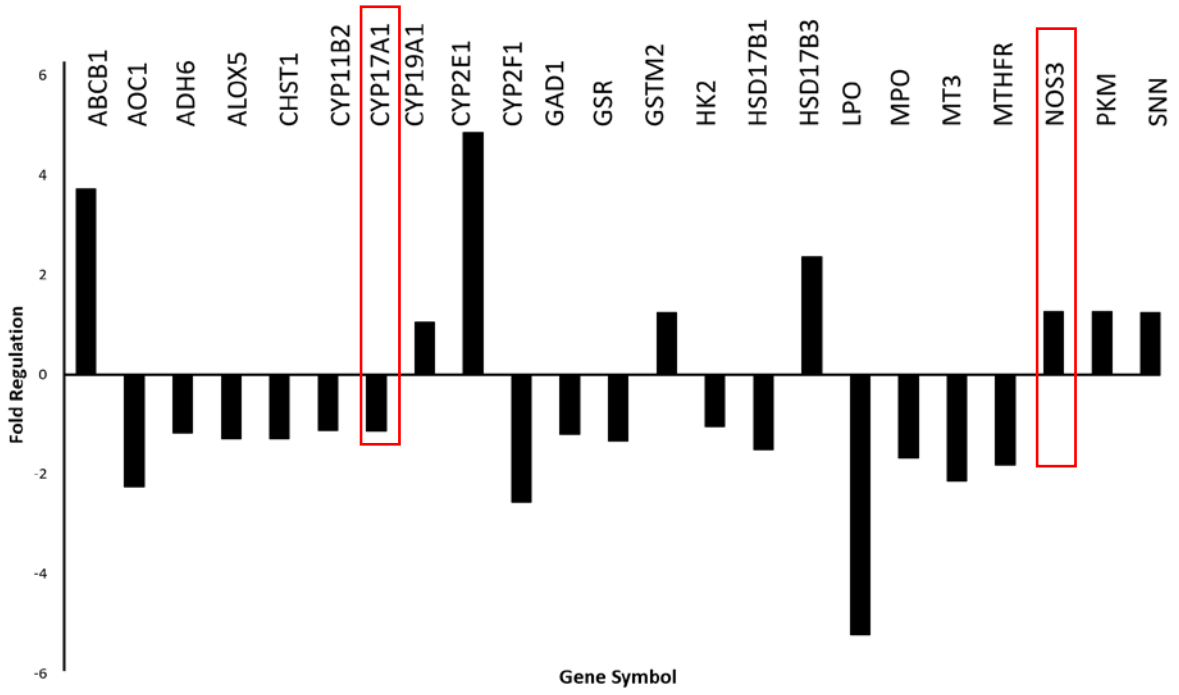


Layout	01	02	03	04	05	06	07	08	09	10	11	12
A	ABCB1 3.52 B	ABCC1 -60.71 A	AOC1 -2.38 B	ADH1B 1.59	ADH1C -21.25	ADH4 10.57	ADH5 -2.66	ADH6 -1.23 C	AHR -2.75	ALAD -40.63	ALDH1A1 5.04	ALOX12 -82.50 A
B	ALOX15 -65267.79 A	ALOX5 -1.36 B	APOE 1.87	ARNT -2.16	ASNA1 1.11	BLVRA -1.21	BLVRB -1.24	CES1 -26.01	CES2 -15.93	CES3 -1.05	CHST1 -1.36 B	COMT 51.94 A
C	CYB5R3 -2.49	CYP11B2 -1.17 B	CYP17A1 -1.20 B	CYP19A1 -1.01 B	CYP1A1 -1.70	CYP2B6 1.02	CYP2C19 -1.24	CYP2C8 -4.18	CYP2C9 -1.15	CYP2D6 -1.01	CYP2E1 4.58 B	CYP2F1 -2.70 B
D	CYP2J2 1.27	CYP3A4 2.69	CYP3A5 -2.69	EPHX1 1.64	FAAH 1.42	FBP1 2.10	GAD1 -1.27 B	GAD2 19.85 B	GCKR -2.52	GPI -1.16	GPX1 1.84	GPX2 -4.81
E	GPX3 -1.71	GPX4 -1.25	GPX5 -2.50 B	GSR -1.41 B	GSTA3 -1.26	GSTA4 -1.30	GSTM2 1.17 B	GSTM3 -1.44	GSTM5 -11.44 B	GSTP1 -2.61	GSTT1 1.23	GSTZ1 -1.07
F	HK2 -1.09 B	HSD17B1 -1.59 B	HSD17B2 -1.48	HSD17B3 2.24 B	LPO -5.52 B	MGST1 -1.42	MGST2 -1.72	MGST3 -2.19	MPO -1.76 B	MT2A -1.76	MT3 -2.25 B	MTHFR -1.91 B
G	NAT1 -1.20	NAT2 -1.10	NOS3 1.20 B	NQO1 1.04	PKLR -1.62	PKM 1.19 B	PON1 -1.37	PON2 -1.26	PON3 -7.10	SNN 1.18 B	SRD5A1 -1.35	SRD5A2 -1.57

(Figure-28) Heat map showing gene expression fold change between the 20HE and placebo group in the context of the profiler array layout. The array layout shows fold regulation magnitude displayed in the heat map. ALOX15 gene (highlighted) showed great fold change difference with significance ($p < 0.05$). CYP17A1 and NOS3 genes (highlighted) were of interest due to their favourable effects of sports performance. Comments A, B or C given to few genes refer to cycle threshold (Ct) interpretation of the software. A; Ct is relatively higher in one group suggesting this gene's expression is lower in this group and the fold change minimum is as reported, B; higher Ct were detected in both groups leading to inability to calculate accurate fold change and C; undetected gene expression due to high Ct in both groups.



(Figure-29) Two-dimensional non-supervised hierarchical clustering of 20HE and placebo group gene expression. Figure shows heat map and dendograms indicating co-regulated genes at a sample or group level.



(Figure-30) Fold regulation of few genes of interest detected in the human drug metabolism array. Representation of genes that were up or down regulated in response to 20HE treatment. Notably NOS3 and CYP17A1.

3.2.3 Human Excretion study

Supplement analysis results

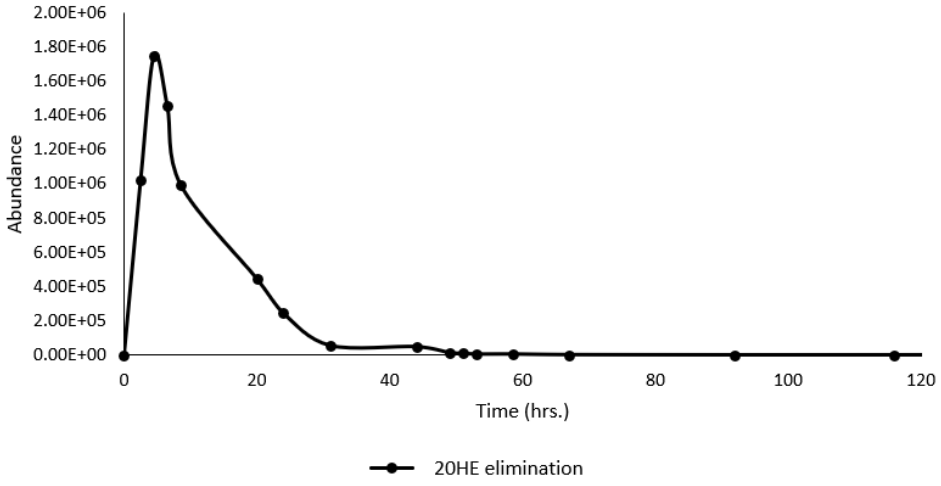
Adding to the human metabolism of 20HE investigation and part of understanding the pharmacokinetics of the drug, excretion studies were conducted. Of importance is that the actual contents of supplements often are not reflected on the label and to overcome this the selected supplements were tested qualitatively and quantitively in-house to quantify 20HE content. In addition, batch-to-batch variability in the supplements was of concern. Therefore, consistency of supplement contents was tested using up to 5 tablets from each supplement lot administered (Table-15)

	Commercial Name	**Quantitation per unit**
1	Turkesterone	2.3mg
2	Ecdybolin	1.008 mg
3	Suma root	176.17 µg
4	Desire X	8.88 µg
5	Immunectar	0.402 µg
6	Natural Sterol extreme	Unquantifiable
7	Medicinal cyathula root	Unquantifiable
8	ZMA PM	Undetectable
9	Beta-X	Undetectable
10	Norateen II	Undetectable
11	Z-Force	Undetectable
12	Bio pro plus	Undetectable
13	MSB methyl plus	Undetectable
14	Power meal	Undetectable
15	Animal Stack (USA)	Undetectable
16	Promax Extreme	Undetectable

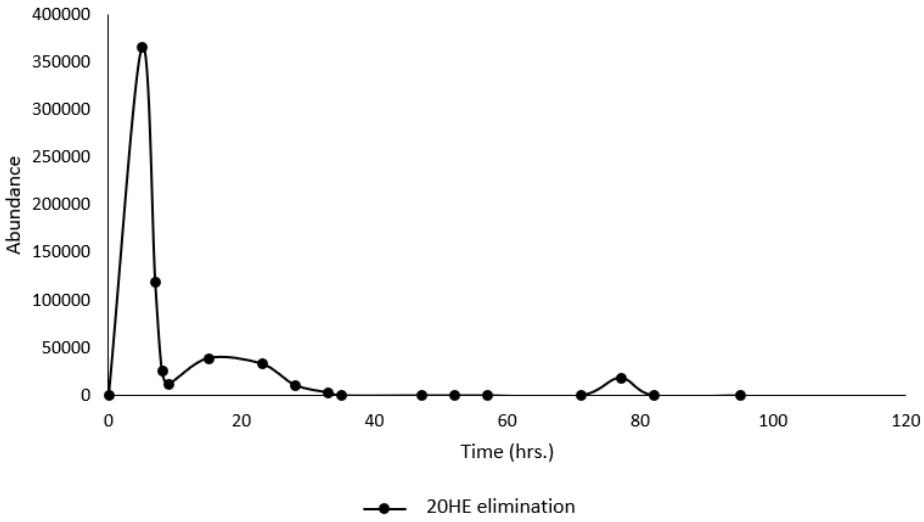
(Table-15) Results from GCMSMS analysis of 16 supplements that claimed to contain ecdysteroids. Only 5 contained detectable and quantifiable amount of 20HE. Two supplements with unquantifiable amount were oil based which may have hindered the analysis.

The excretion study was conducted on a single volunteer taking Turkesterone (20HE) supplement and multiple urine samples were collected on day 1 of the experiment up to day 5 and analysed using GCMS. The parent compound; 20HE was first detected after 2 hours post administration with maximum level detected at 4-5 hours post administration. The detectability of the parent compound remained up to 48 hours post administration of the mislabelled supplement Turkesterone which upon analysis was found to consist of 20HE (Figure-31a). After the washout period excretion study was repeated using Desire-X supplement which contained lesser concentration of 20HE (8.8 µg) where the parent compound remained detectable up to only 36 hours (Figure-31b).

a)



b)



(Figure-31) Urinary excretion profile of 20HE from a single volunteer using GCMS analysis. y-axis represents the abundance which based on signal intensity of the 20HE graphed against time (x-axis). Panel (a) shows the excretion profile of 20HE after administration of Turkesterone supplement and maximum abundance of 20HE was observed 4-5 hours post administration. Panel (b) presents 20HE excretion profile after the administration of Desire-X supplement containing lower level of 20HE where it was observed that 20H was detected up to 36 hours opposed to 48hours observed after administration with Turkesterone in panel (a).

3.3 Discussion of results

Urinary metabolites of Methandienone

In this study the principals of pharmacokinetics were applied to investigate metabolism and excretion of Ecdysteroids. Firstly, the ability of the selected humanised liver murine model to generate the human hepatic metabolites of the gold standard doping agent, Methandienone, was determined. Human hepatic drug responses were compared using data acquired from human and mouse drug metabolism arrays. The experiment was setup to allow the investigation of the sensitivity of the model towards a single dose administration in comparison to multiple dosing and its effect on increasing the sensitivity of detection of human hepatic metabolites of the drug. This study provided an indication of successful in-house detectability of the expected reported metabolites of Methandienone using the analytical method developed and approved by the world anti-doping agency (WADA) to be used for the detection doping incidents from human urine samples obtained from athletes.

Profiler array analysis

Mouse phase I drug metabolism enzymes profiler array was used to investigate the hepatic response of the residual mouse liver in the chimeric mice and this was compared to human phase I drug metabolism profiler array. This investigation further confirmed the suitability of the model for the detection of 20HE metabolites and lead to the next aim of this project to investigate the human hepatic metabolism of 20HE using the uPA +/+ SCID mice model.

Aldehyde dehydrogenase activity in blood

After the global upregulation of ALDH family of enzymes observed in the profiler array analysis further confirmation of its activity in the blood was investigated. No significant changes were observed in the plasma. The most apparent limitation faced in the investigation was the use of plasma and for future investigation serum may be better suited for this investigation.

Urinary metabolites of 20HE

Results from the first experiment using uPA+/+ SCID mice to generate human hepatic metabolites of Methandienone provided a solid ground to build the next experiment using the same murine model for the detection of human hepatic metabolites of 20HE using multiple dosing which increased the sensitivity of detection in the first experiment. One of the drawbacks of this model was the small volume of urine collected from these mice throughout a 24hours period.

Profiler array analysis

Hepatic gene expression profile after administration of 20HE was investigated using human drug metabolism profiler array. Results indicated the upregulation of 15 genes and down regulation of 29 in the 20HE opposed to the placebo group. Genes involved in steroidogenesis regulation and vascular function (CYP17A1 and NOS3 respectively) that were significantly differentially expressed were of interest. Since 20HE has been added in the WADA monitoring list due to its purported these genes presented basis for future studies in the context of doping and performance enhancement.

Human Excretion study

Further investigation of 20HE excretion was conducted on one individual using selected 20HE containing supplement and another that does not contain 20HE. To overcome the extensive process of ethical restrictions in this study, a dietary supplement containing 20HE was used, since these supplements reach the market directly from manufacturers without the need of approval from any regulatory body; as is the case for drugs. 20HE was first detected 2 hours after administration with peak detection intensity between 4-5 hours which decreased but remained detectable up to 48 hours. Lower concentration showed 20HE excretion period up to only 36 hours. These parameters are key in developing methods to detect the use of this compound in case 20HE is moved to the WADA prohibited list.

CHAPTER 4

RESULTS●PHYSIOLOGICAL EFFECTS OF ECDYSTEROIDS

Dietary supplements intake in addition to being globally wide spread, its use is prevalent among elite athletes. Meta data analysis studies revealed that elite athletes use dietary supplement more often than recreational athletes (11). Reasons behind this prevalence of use is that among athletes include compensation for specific deficiencies such as iron or vitamin E where it was reported that female athletes often use the former while male athletes consume the later or protein and creatine (11). Other reasons relate to sport performance enhancement by increasing muscle recovery and training effectiveness and reducing risk of muscle injury as explained by the international Olympic Committee IOC in the supplement consensus statement published in 2017 (22). Out of 16 supplements claiming having 20HE, two were chosen for this this study based on their concentration of 20HE or lack thereof (Desire-X and Z-Force respectively). A cohort of recreational athletes was recruited and their anthropometric, blood pressure and aerobic fitness measurements were recorded before and after 2-months intervention with the supplements. Measures of aerobic fitness were further investigated *in-vitro* testing mitochondrial membrane potential after overnight treatment with different concentration of 20HE in human skeletal cells myoblasts and preadipocytes. Transcriptome and proteomics data were also conducted using extracted RNA from livers of the uPA+/+ SCID murine model treated with 20HE or placebo.

4.1 Human Studies

4.1.1 *in-vivo* studies

Previous reports showed the anabolic activity of 20HE when taken at high concentrations (54-56,66). Here the focus was on supplement derived 20HE intake at a lower concentration using 20HE containing supplement compared to a supplement that does not contain 20HE. This supplement though it is recommended by the manufacturer to take 2 to 3 tablets per day, the total concentration was considered lower than the levels that were reported as anabolic. This study provides insight on 20HE effects at low non-anabolic concentration on body composition, blood pressure, blood chemistries and aerobic fitness.

4.1.1.1 Body composition

Changes in Body Composition of the recruited recreational athletes were measured before and two months after the intervention with 20HE supplement and is summarized in (Table-16). Though the two groups were not age matched, this was not considered a limitation since the comparison was conducted before and after the intervention in the same individual and no comparison between the groups was conducted. A slight increase in body weight in the 20HE supplement group was observed after the intervention explained by increase in muscle mass with actual decrease in fat mass (Table-16). This increase is behind the noticeable increase in BMI after 20HE intervention.

Intervention supplement	Age (yrs)	Height (m)	Time point	Weight (Kg)	BMI (kg/m^2)	Fat mass (Kg)	Muscle Mass (Kg)
Desire-X (20HE)	41.71 (8.94)	171.29 (2.91)	Visit 1	73.86 (6.78)	24.94 (2.44)	15.50 (3.86)	55.43 (3.55)
			Visit 2	74.21 (5.78)	25.09 (2.88)	15.10 (4.74)	56.14 (2.88)
Z-Force	51.5 (13.6)	173.17 (2.61)	Visit 1	76.70 (8.24)	25.87 (2.27)	17.65 (4.74)	56.10 (4.14)
			Visit 2	76.35 (8.36)	25.77 (2.76)	16.75 (4.15)	57.05 (4.58)

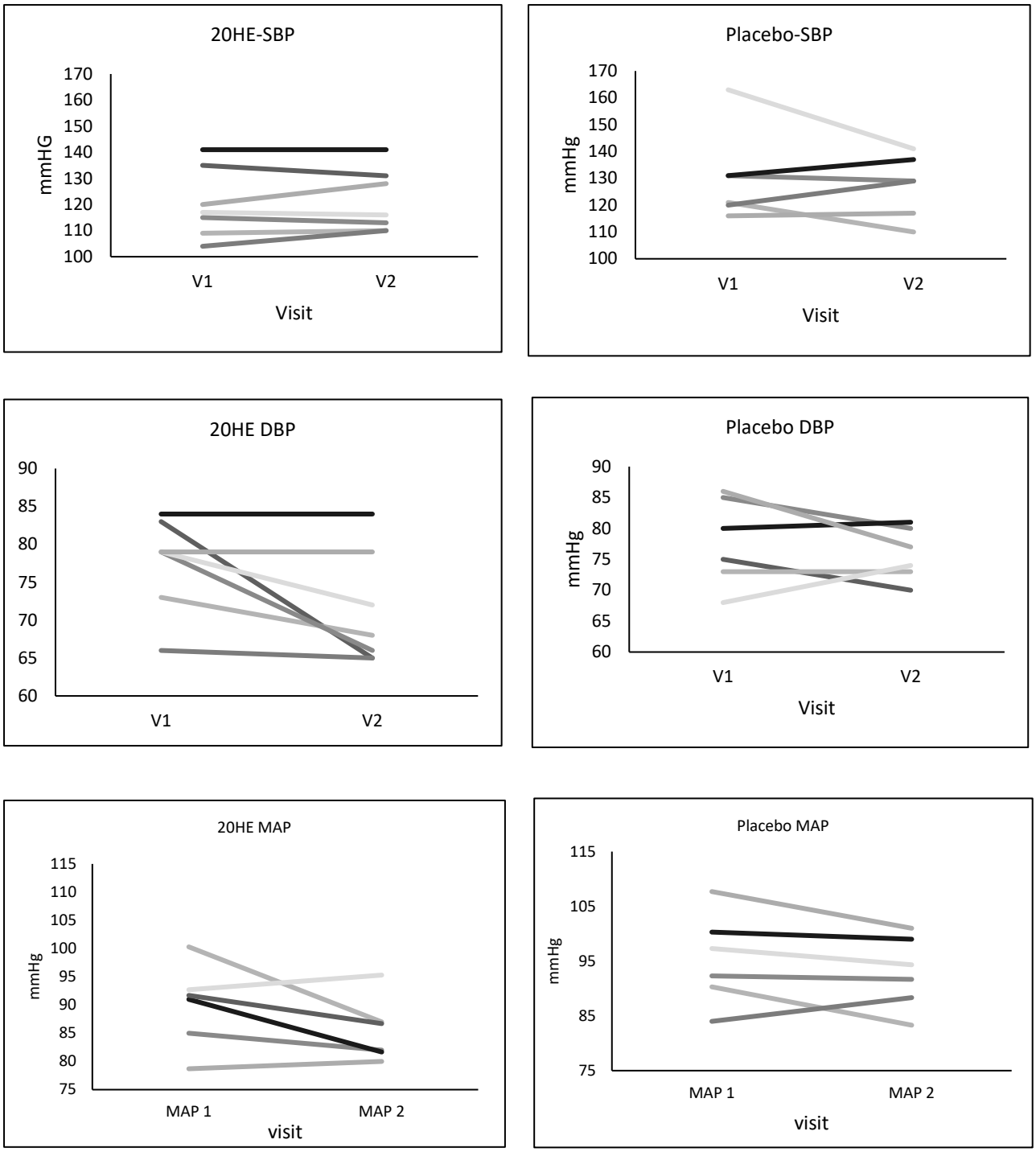
(Table-16) Whole body segmental analysis measured on recruited recreational athletes before and after supplement intervention using. No significant changes in muscle mass and fat mass in the 20HE group were observed. Data shown as mean (standard deviation).

4.1.1.2 Blood

Blood Pressure measurements were recorded before and after the intervention (Table-17). Significant decrease in the diastolic blood pressure (*p*=0.04) was observed after 20HE supplement intervention. No other significant changes were noted. Individual changes in measures of blood pressure before and after the interventions are presented in (Figure-32)

	Desire-X (20HE) (n=7)			**Z-Force (n=6)**		
BP measure	**Visit 1**	**Visit 2**	***p*-value**	**Visit 1**	**Visit 2**	***p*-value**
SBP (mmHG)	120 (13.3)	121 (12)	0.51	130 (17)	127 (11.7)	0.53
DBP (mmHG)	77.6 (6.2)	71.3 (7.5)	**0.05**	77.8 (7.1)	75.8 (4.6)	0.40
MAP (mmHG)	91.8 (8.3)	87.9 (8.4)	0.13	95.3 (8.3)	92.9 (6.6)	0.22

(Table-17) Changes in blood pressure prior to and after 20HE or placebo supplementation. Significance was observed only in the diastolic blood pressure of 20HE-supplement taking group with *p*-value= 0.05. Results reported as mean (standard deviation).



(Figure-32) Effect of Desire-X; a 20HE containing supplements on blood pressure. Recorded blood pressure measurements of systolic (SBP) (top panel) Diastolic (DBP) (middle panel) and mean arterial (MAP) (bottom panel) blood pressures of each individual in both groups assigned for 20HE supplement or placebo.

Haematology report from Sysmex indicated a significant drop in RBC count (p=0.01) thus the significant drop in haematocrit to 41.5% (p=0.04). This was also observed in platelets count but failed to reach significance (p=0.07). Creatinine levels were significantly elevated after the intervention with 20HE supplement (p=0.03) which may indicate lower glomerular filtration rate in the kidney. Changes in systemic glucose and lipids were recorded and summarised in (Tabl-18). For the group consuming 20HE containing supplement (Desire-X) a marginal decrease in glucose, HDL cholesterol and triglycerides were observed but non reached significance. Similar profile was observed in the placebo supplement group (Z-Force) for the glucose and total cholesterol. Interestingly, significant decrease in the healthy cholesterol (HDL-Cholesterol) was recorded in response to Z-Force p= 0.03 (Table-18).

Supplement	Desire-X (20HE)			Z-Force		
Time point	**Visit 1**	**Visit 2**	***p*-val**	**Visit 1**	**Visit 2**	***p*-val**
Glucose	5.20 (0.19)	5.05 (0.64)	0.75	5.48 (0.52)	5.30 (0.55)	0.33
Total Cholesterol	5.24 0.90	5.30 (0.64)	0.93	4.70 (1.49)	4.62 (1.32)	0.45
LDL-Cholesterol	3.36 (0.81)	3.79 (0.52)	0.38	3.00 (1.35)	3.20 (1.32)	0.07
HDL-Cholesterol	1.72 (0.40)	1.54 (0.22)	0.26	1.56 (0.28)	1.39 (0.17)	0.03
Triglycerides	1.00 (0.29)	0.91 (0.29)	0.22	0.84 (0.39)	0.97 (0.27)	0.29

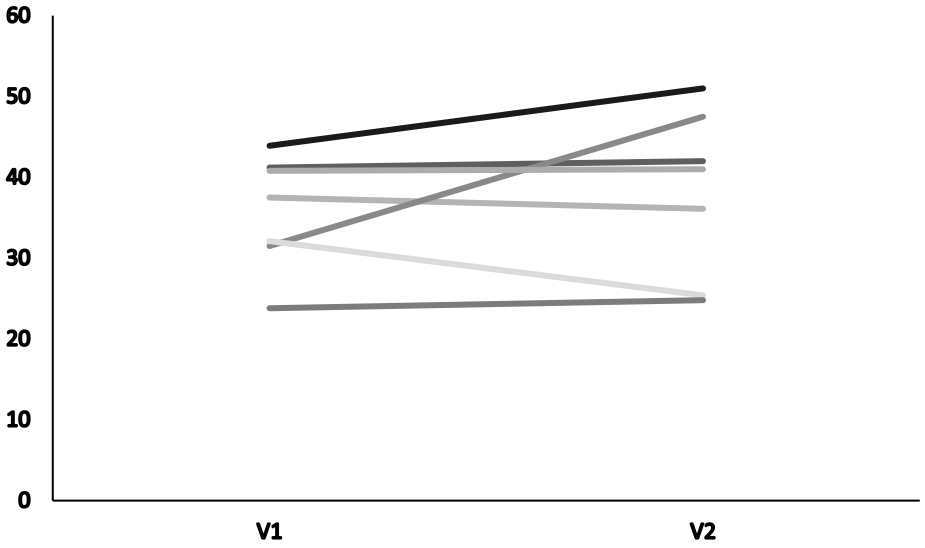
(Table-18) Recorded systemic glucose and lipids for both groups. Glucose levels were marginally reduced in both groups and comparing both groups no other significant changes were observed. Significant decrease in HDL cholesterol was observed after intervention with the placebo supplement with p-value= 0.03.

4.1.1.3 Aerobic fitness

Basic level of aerobic fitness was recorded during the initial visit and then recorded again after the intervention. Results are summarised in (Table-19). Improvements in aerobic indices were seen after the intervention with 20HE supplement. A 1.10 fold increase in VO_{2max} was observed after 20HE intervention (Figure-33) and the velocity at VO_2 (vVO_2) which refers to the pace at which the VO_{2max} was achieved was also improved after the intervention with 20HE supplement. Also, metabolic equivalent (MET) was increased after the intervention with 20HE containing supplement meaning the energy expenditure has increased indicating increased aerobic fitness. These indices of aerobic fitness were actually decreased in the placebo supplement group.

Maximum voluntary ventilation MVV is a measure of gas exchange that describes the air volume an individual breathes as quickly and deeply as possible in a given time frame. This measure reflects ventilatory function while respiratory quotient RQ represents the respiratory exchange ration RER which during resting state is equal to the RQ. RQ value indicates the energy consumed for metabolic processes where when carbohydrates are the primary source of energy for the metabolic process RQ value is equal to 1 and a value of less than 1 indicates a mixture of carbohydrates with proteins or fat (RQ=0.8 or 0.7 respectively) (88). MVV and RQ measurements were significantly reduced (p <0.01) after the intervention with 20HE while in the placebo supplement group MVV was significantly reduced (p <0.01) and RQ was also reduced without statistical significance.

Aerobic exercise directly relates to aerobic respiration process in the mitochondria for the continuous energy generation. The mitochondrial respiration is indicated by the polarisation within the compartments of each mitochondrion i.e. mitochondrial membrane potential MMP which was investigated in the following section.



(Figure-33) Change in VO_{2max} before (V1) and after (V2) intervention with 20HE supplement. One data set showed a decrease in VO_{2max}, which after urinary investigation indicated the individual stopped ingesting the supplement.

	Desire-X(20HE)			Z-Force		
Variables	**Visit 1**	**Visit 2**	***p*-val**	**Visit 1**	**Visit 2**	***p*-val**
VO_2 max	34.42 (7.17)	37.94 (10.97)	0.41	33.53 (9.74)	33.32 (7.61)	0.88
VE/VCO_2	40.86 (15.33)	30.22 (3.41)	0.04	29.35 (3.82)	34.90 (10.81)	0.33
vVO_2	9.83 (2.05)	10.84 (3.13)	0.41	9.58 (2.78)	9.52 (2.17)	0.89
MET	9.86 (2.07)	10.86 (3.13)	0.42	9.57 (2.77)	9.52 (2.19)	0.90
MVV	140.22 (11.71)	140.08 (11.75)	<0.01	134.83 (12.31)	134.60 (12.33)	<0.01
RQ	1.14 (0.11)	0.99 (0.07)	<0.01	1.19 (0.09)	1.14 (0.12)	0.46

(Table-19) Recorded measures of aerobic fitness before and after supplement intervention. Overall improvements in aerobic indices after 20HE supplement intervention were observed. Data are shown as mean (SD). Significance derived from Student's t-test, with $p<0.05$ being significant.

4.1.2 Human *in-vitro* study: Effect on mitochondrial membrane potential (MMP)

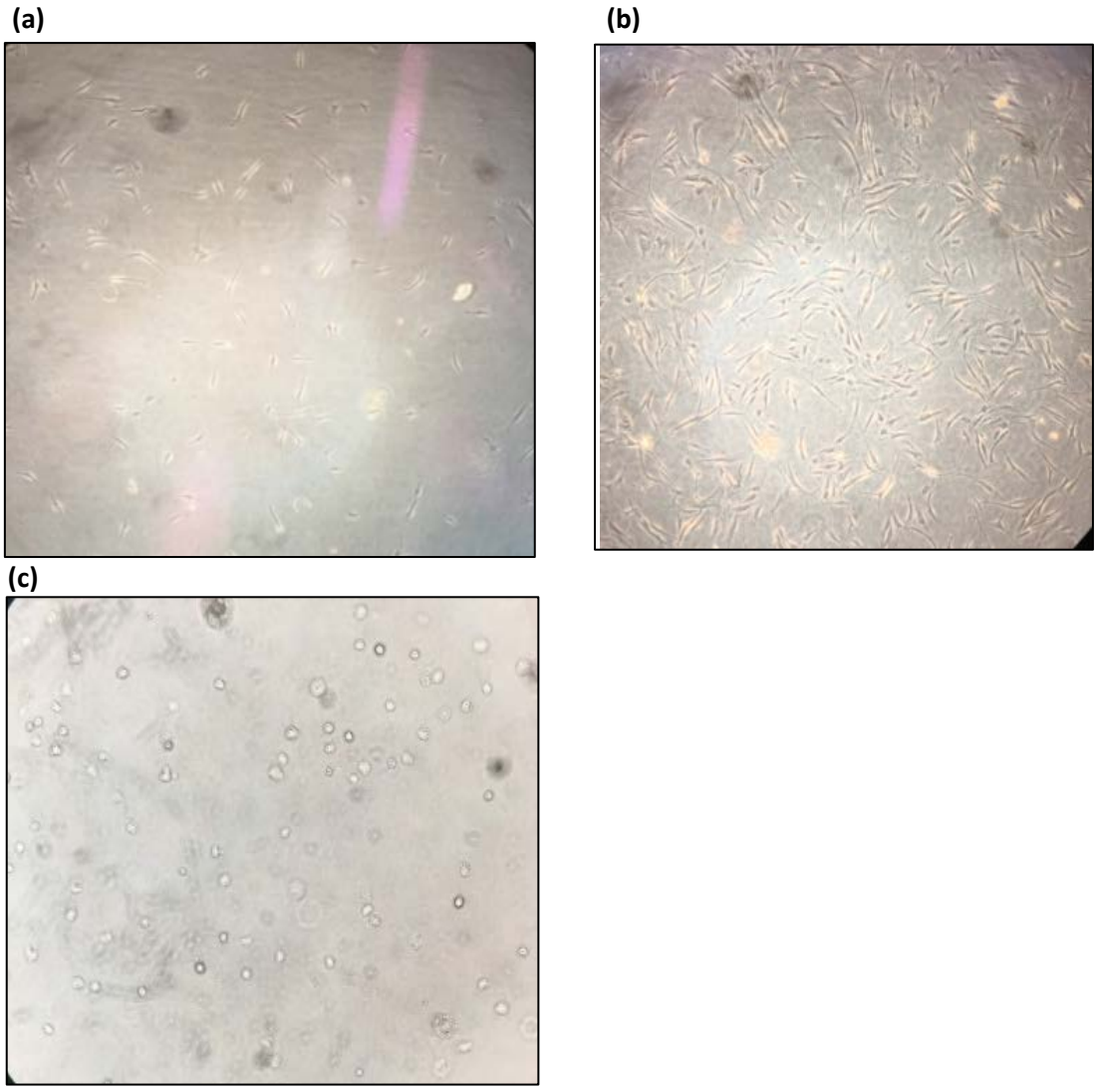
In this section the aim was to investigate aerobic respiration of mitochondria in response to 20HE since improvements in aerobic fitness indices were observed after *in-vivo* investigation. Increasing concentrations of 20HE were used to treat human preadipocytes and myoblasts to identify the effect of 20HE on mitochondrial polarisation or mitochondrial membrane potential (MMP).

4.1.2.1 Preadipocytes

Cell culture optimisation

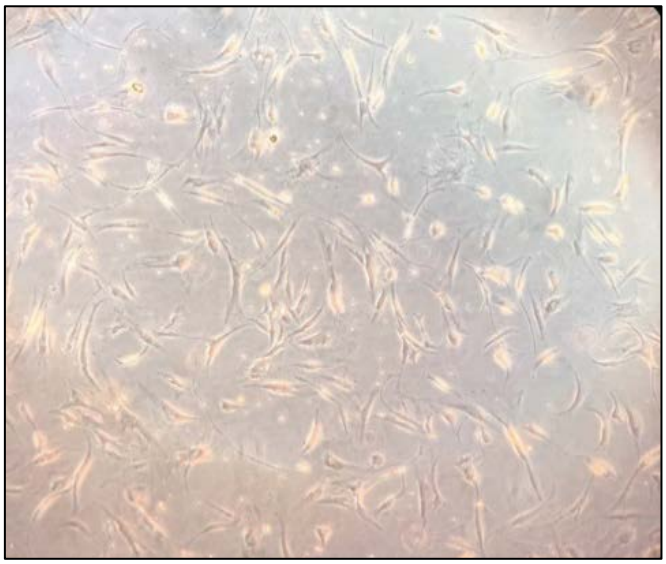
Lean human White Preadipocytes (HWP) from subcutaneous depots at passage 6 (P6) were resurrected according to the manufacturer's protocol and seeded in two T25 flasks. At 80% confluence the cells were passaged to two T75 flasks. Cells were growing at a slower rate than before the passage. It was thought to be due to passaging at 80% confluence or the low numbers of cells that were seeded in each flask. Juxta-positioning of the cells may have been an important factor and due to the unusual slow growth of cells, they were trypsinised back to two T25, using 3mL trypsin (just enough volume to cover all surface area of the flask) and then incubated at 37^{o}C with 5% CO2 for 2-5 minutes. It was observed that after 3 minutes all cells were in suspension. All 6mL of the trypsinised cells from both flasks were added to 6mL of growth media in 15mL falcon tube. Cells were pelleted by centrifugation at 1200 RPM for 5minutes at room temperature and all media and trypsin was pipetted out. Cells were washed with 5 mL of media and centrifuged at the same conditions to remove any traces of trypsin. Cells were then resuspended in 4mL media and seeded into two T25 flasks. Unfortunately, cells did not survive the transfer to the T25 (Figure-34). Lessons learnt from these initial experiments were 1) Cell number should be adequate when seeding on the first day, 2) cell-to-cell contact greatly improves cell growth, and, 3) the cells should only be passaged when they are at 100% confluence.

A new aliquot of primary human white preadipocytes (HWPA) was resurrected into T-25, since the previous aliquot had very low cell counts, and grew very poorly during a three-week period. Media was changed 24 hours after resurrection. There appeared to be more cells that had adhered compared to the previous attempt, which were at about 20% confluent 24 hours after seeding. Following this, cells were observed every day and the media changed every 48 hours. Cells were about 30% confluent which was still relatively slow. A possible explanation for this could be that these were commercially available primary cells, with very limited passaging capabilities. Further, the donor of these cells was a lean Caucasian middle aged female (45 years). Cells from older subjects often lose their plasticity and ability to replicate sooner than those from young donors. Cells were at 30% confluence and apparently were senescent but were still maintained to see if they would proliferate. Results from these cells may understandably be unreliable. All these initial aliquots were resurrected in T25 flasks but after a month of senescent cells and slow proliferation; cells were resurrected in 6 well plates instead (Figure-35). This protocol was adopted for future experiments for RNA and protein extraction.

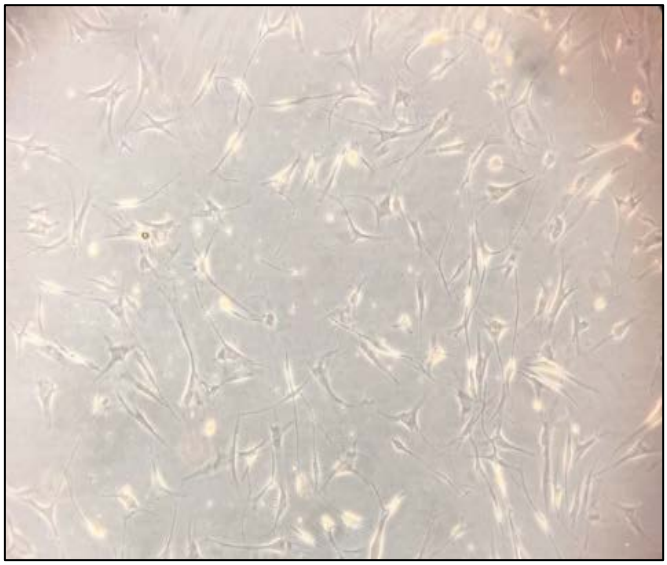


(Figure-34) Progression of preadipocytes growth. There were major difficulties in the initial studies with the growth of preadipocytes. Cells 24hours after seeding on T75 flask (a), cells at 80% confluence (b) which were passaged to T25 flask and after 24 hours the cells did not survive (c).

(a)



(b)



(Figure-35) Cells resurrected and grown on 6-well plates to overcome the low viability rate after resurrection in T25 flasks. Cells 24 hours after initial resurrection and seeding (a) and observed cell growth 48 hours after the first passage (b).

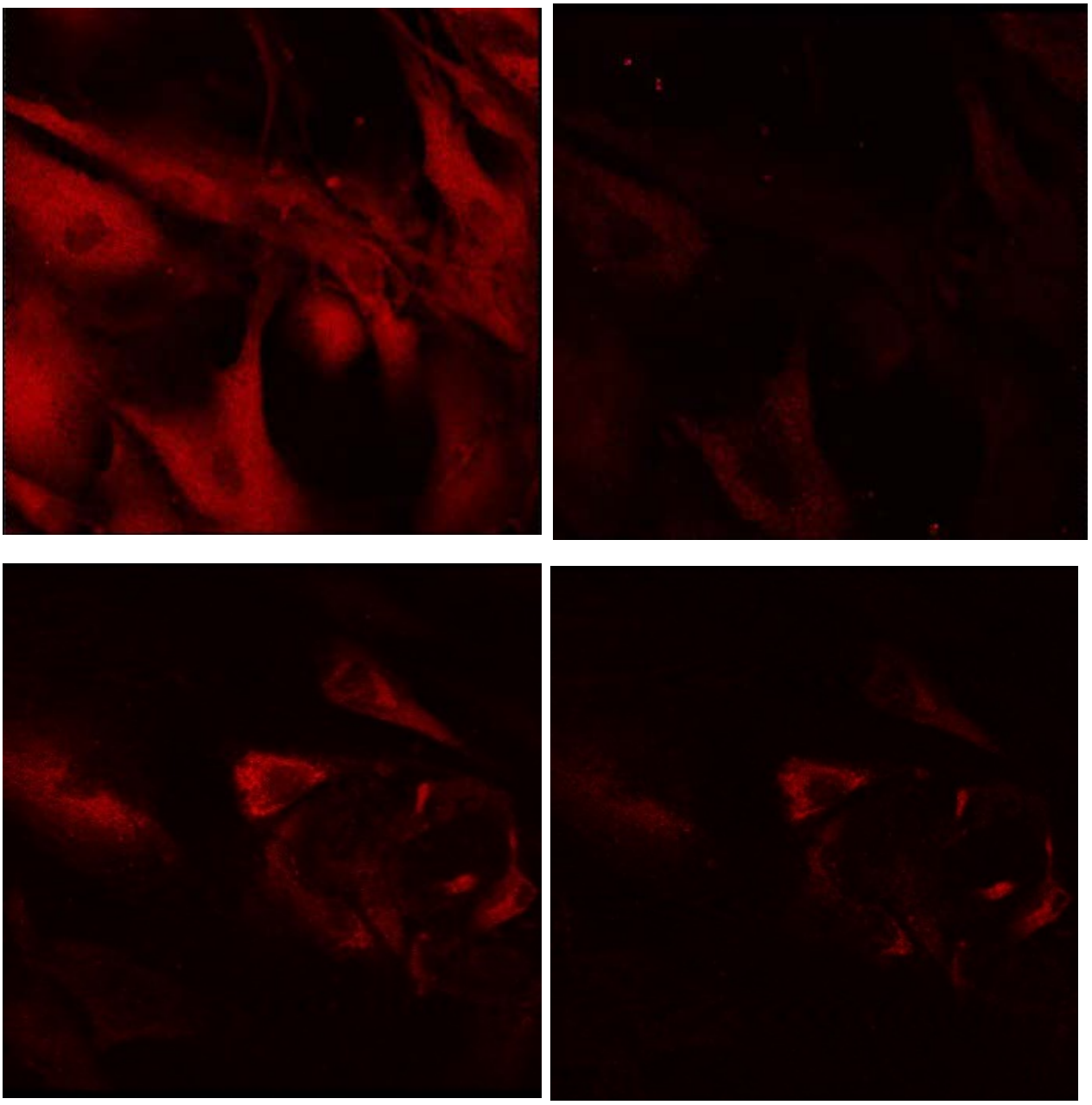
Series of experiments were carried out to test the best seeding densities for the microscope coverslips in order to commence the planned confocal studies. The first attempted seeding density was 1500 cells and after overnight incubation at 37^{o}C/5% $CO_{2,}$ cells were sparse and mainly seen at the edges of the cover slip. This was probably due to the low number of cells seeded and the positioning of the cover slip inside the 24-well plate. On the second attempt cells were seeded on three additional cover slips each at density of 5000 cells which proved to be too high though individual cells were successfully visualised, the fields were overcrowded. Thus, the next batch of coverslips were seeded with 2500 cells which was the best seeding density and it was used for the experiments that followed as standard procedure.

Confocal imaging

An initial experimental plan included the cells grown on coverslips for confocal studies and in 6-well plates for RNA, DNA and protein extraction. Three coverslips were seeded with preadipocytes and after overnight incubation one coverslip was treated with the highest dose of 20HE (10µM) overnight while media was changed for the remaining two coverslips. All coverslips were investigated under confocal microscopy after TMRE staining and the delta change in the intensity was recorded after the addition of FCCP (Figure-36). The initial studies showed that the TMRE concentrations used were able to capture good quality images of the mitochondria, with increased intensity, suggesting coupled mitochondria. After the establishment of the optimal conditions additional experiments were conducted with treatment with 0, 1 ,5 and 10 µM concentrations of 20HE.Results from the combined protocol for the extraction of RNA, protein and DNA from these cells are shown in (Table-20).

Sample	Sample Type	Conc. (ng/µL)	260/280	260/230
C1	DNA	62.5	1.49	1.29
C2	DNA	60.8	1.44	0.91
C3	DNA	28.5	1.48	0.23
D1	DNA	54.6	1.45	1.15
D2	DNA	37.5	1.46	1.08
D3	DNA	26.5	1.55	1.04
Sample	**Sample Type**	**Conc. (µg/mL)**	**A280**	**260/280**
C1	RNA	1639	1.94	1.03
C2	RNA	1105.7	1.76	0.44
C3	RNA	1510.5	1.89	0.77
D1	RNA	996.3	1.72	0.38
D2	RNA	990.7	1.72	0.38
D3	RNA	1516.3	1.88	0.73
Sample	**Sample Type**	**Conc. (µg/mL)**	**A280**	**260/280**
C1	Protein	48	0.048	0.60
C2	Protein	73	0.073	1.94
C3	Protein	366	0.366	1.29
D1	Protein	280	0.280	1.09
D2	Protein	638	0.638	0.99
D3	Protein	624	0.624	0.90

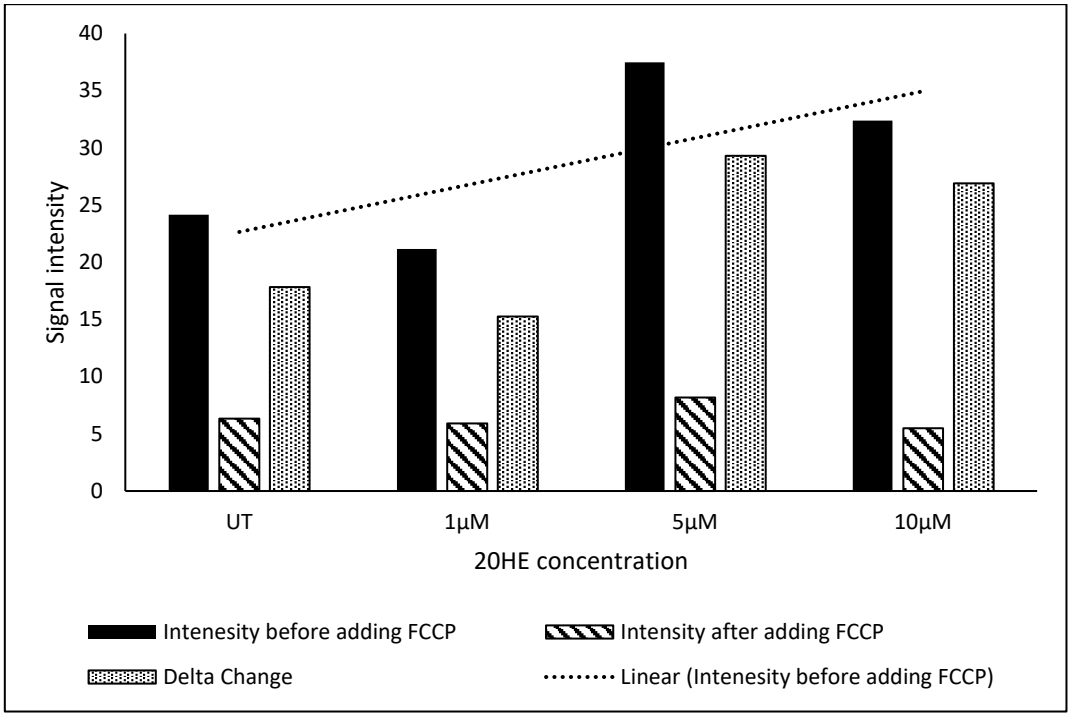
(Table-20) Results of DNA, RNA and protein extraction from cells using a combined protocol. The top panel shows the quantity and quality of the DNA and RNA, and the bottom panel the protein, extracted ascertained by nanodrop.



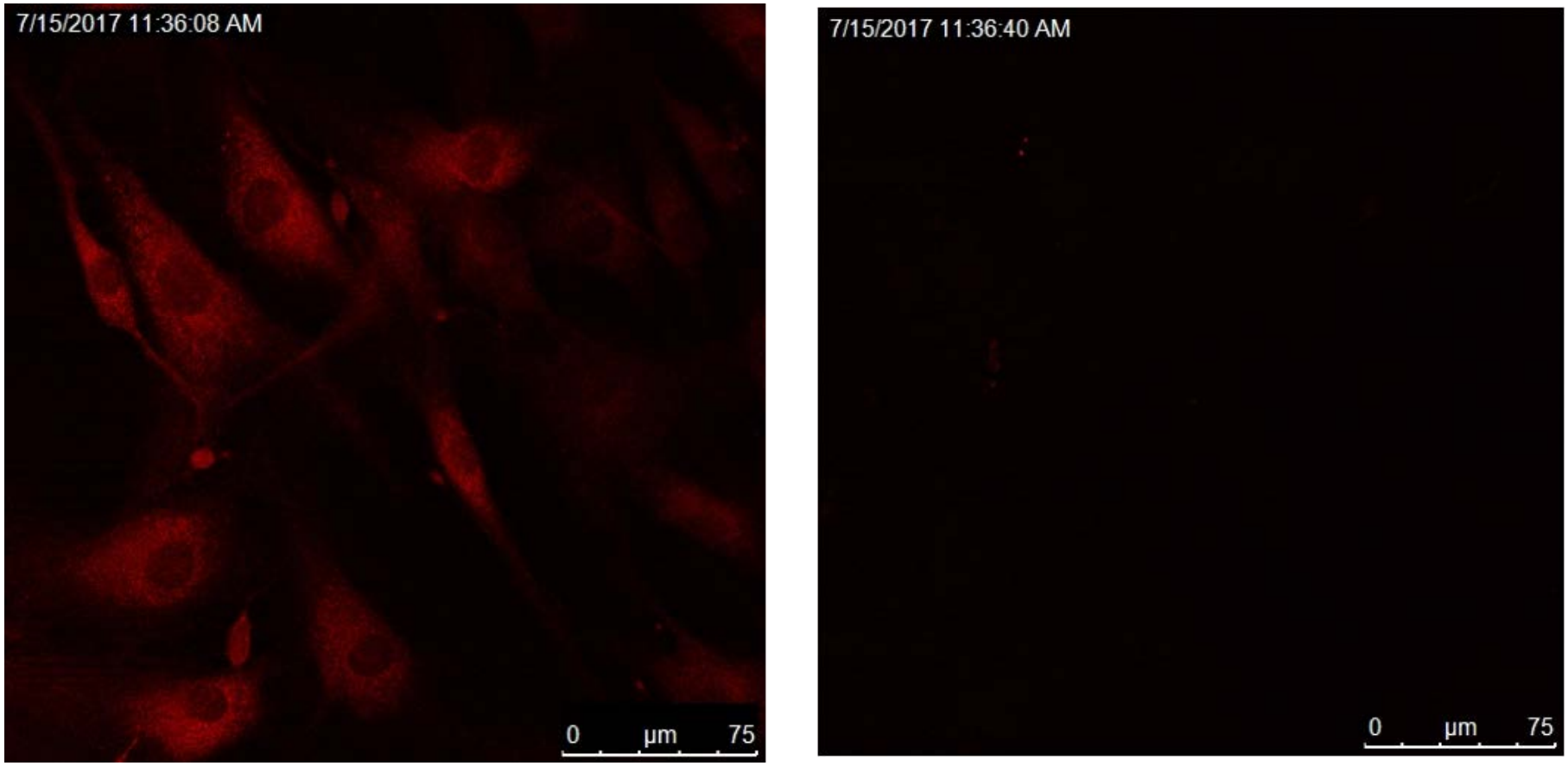
(**Figure-36) Visualisation of initial confocal studies conducted on preadipocytes before and after adding MMP uncoupler**. Preadipocytes were seeded on coverslips and treated with 10µM 20HE (top panel) or vehicle (bottom panel) for 24 hrs. Delta change was calculated based on the intensity before (left) and after (right) treatment with FCCP where the calculated delta changes after 20HE treatment was 37.28 and 7.39 for the placebo treated cells.

Experiments with increasing doses of 20HE

The same format used in the previous section was conducted to test the effect of 20HE treatment on MMP at doses of 0, 1, 5 and 10 µM. Results from two experiments conducted on preadipocytes each consisting of four coverslips treated with 0, 1,5 & 10 µM 20HE overnight are shown in (Figure-37). No significant change was observed between the untreated and 1µM treated preadipocytes in the delta change of MMP intensity after the addition of FCCP (Figure-38). Significant increases in delta change were observed in the 5 & 10µM treated preadipocytes compared to the untreated cells.



(Figure-37) Preadipocytes confocal results averaged from two experiments. Observed difference in mitochondrial membrane potential after treatment with 20HE. Significant increase in MMP in the 5 and 10 µM treated cells compared to the untreated cells. No significant difference in MMP was observed after treatment with 1µM 20HE.



(Figure-38) Effect of FCCP; mitochondrial membrane potential uncoupler on TMRE stained preadipocytes. Preadipocytes before (left) and after (right) adding FCCP. Maximum amplitude on intensity was recorded from the field before adding FCCP and then the minimum amplitude recorded after the addition of FCCP was subtracted giving the delta change in MMP.

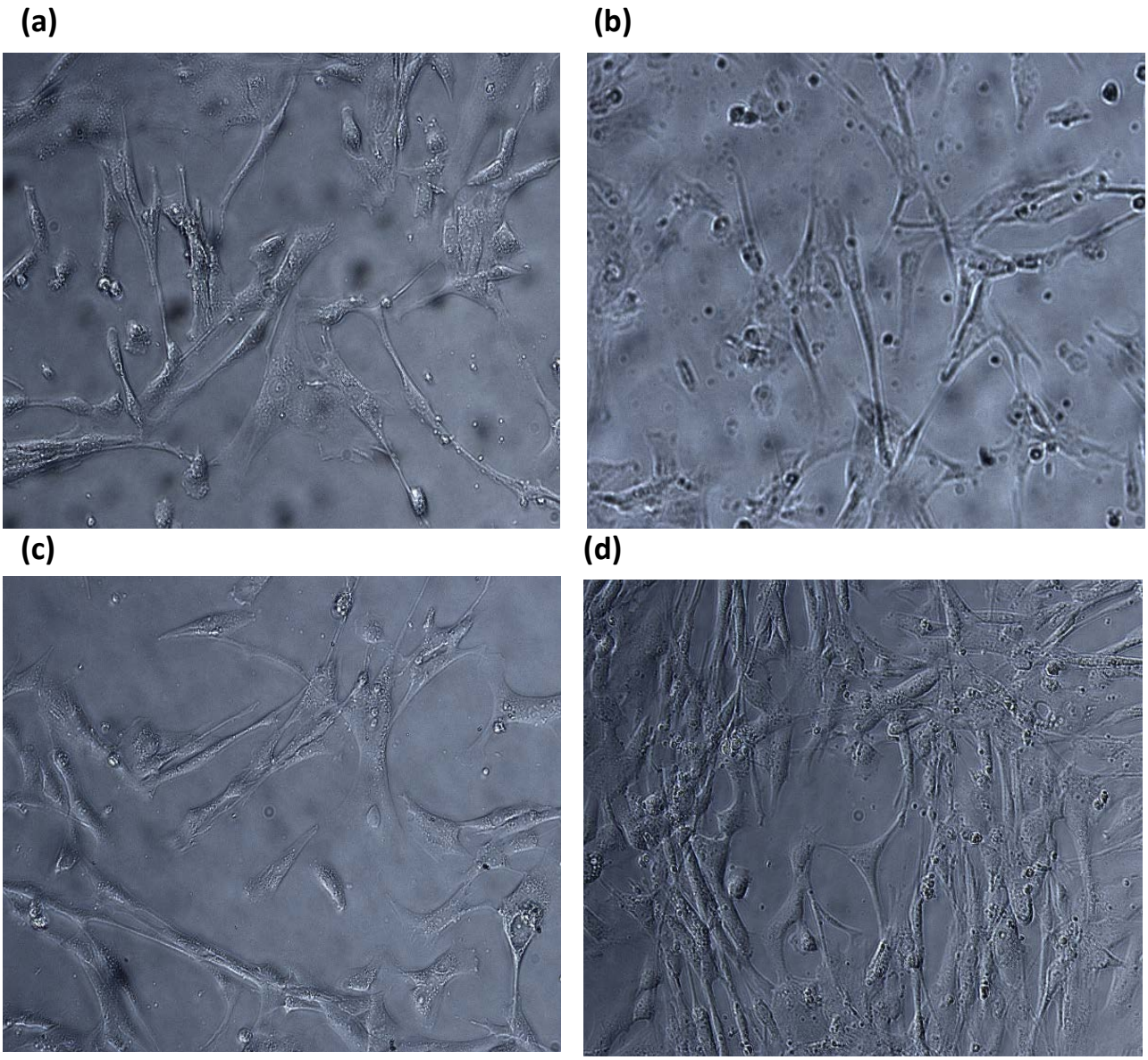
4.1.2.2 Myoblasts

Seeding density optimisation

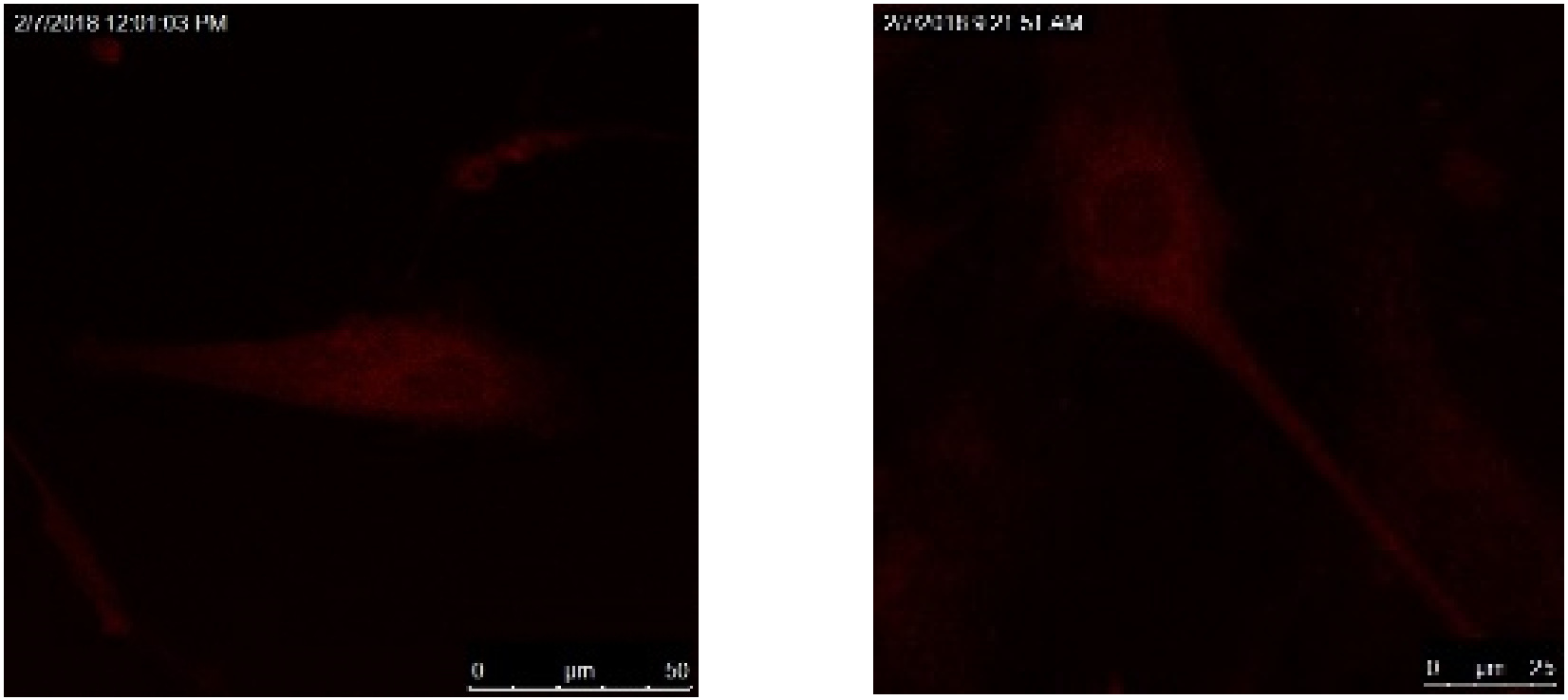
To begin investigating MMP on myoblasts, seeding density optimisation study similar to the preadipocytes optimisations were conducted. chamber slides were also tested for suitability in this section due to their practicality when compared to coverslips.

Use of cover slips compared to 4-well chamber slides

Seeding density optimisation on the 4-chamer slide showed that seeding at a density of 20,000 cells per chamber yielded fields that were too crowded causing cells to overlap. The best seeding density was at 10,000 cells per chamber (Figure-39). Cells were also seeded on cover slips for comparison reasons of both slide types (coverslips or chamber slides) according to the protocol detailed in previous section. No differences in visualization of cells were observed (Figure-40). Advantages of chamber slides included: practicality, ability to culture the cells for few days on the slides which meant more experiments can be conducted per week and the ability to differentiate the cells when they reach confluence on the slide without the need to trypsinise and seed into coverslips. The main disadvantage or difficulty was faced during the addition of FCCP where the well walls prevent the proper placement of the pipette tip especially since FCCP was added in a relatively small amount (10µl) in comparison to TMRE stain (500µl). Other parameters such as the density of glass when using coverslips placed on a slide in comparison to the density of glass of the chamber slide, had not have any noticeable differences (Figure-40).



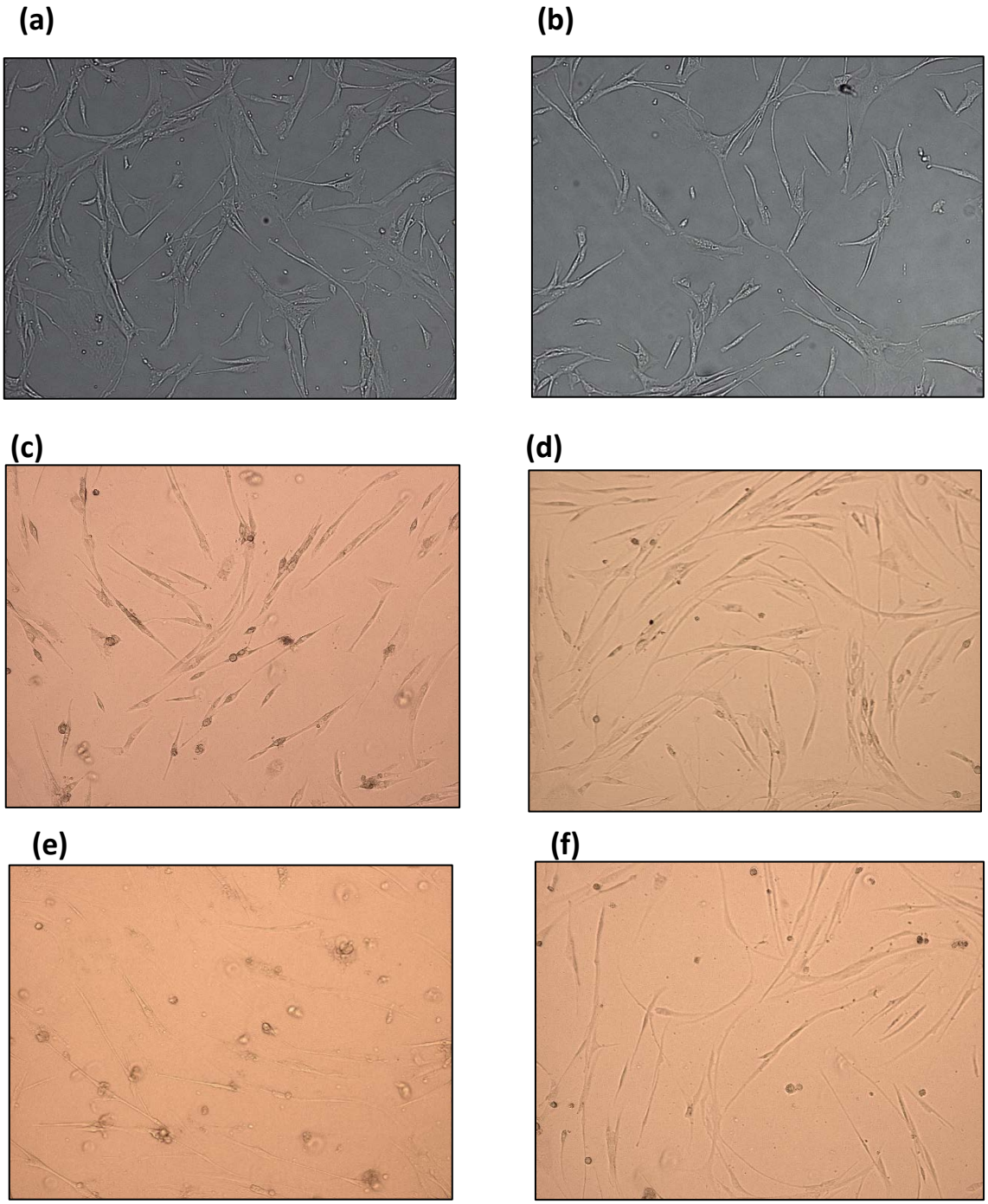
(Figure-39) Myoblasts seeding density optimisation in 4-well chamber slide. 5000 cells (a), 10,000 (b) cells, 15000 (c) cells and 20,000 (d) cells. The density of 10,000 cells for seeding was chosen for all subsequent experiments (b).



(Figure-40) Comparison on visualisation of myoblasts grown on cover slips (left) and 4-well chamber slides (right). No visual differences were noticed, comparing coverslips and chamber slides.

Media comparison

Optimising the use of DMEM as a replacement of manufacturer's recommended media revealed that myoblasts were able to grow in both media but in a slower rate in DMEM up to 4 days where the cells growing in DMEM were senescent and by day 8 cells were dead and the experiment was terminated (Figure-41). As a result, DMEM cannot replace manufacturer's media and only the latter was used.

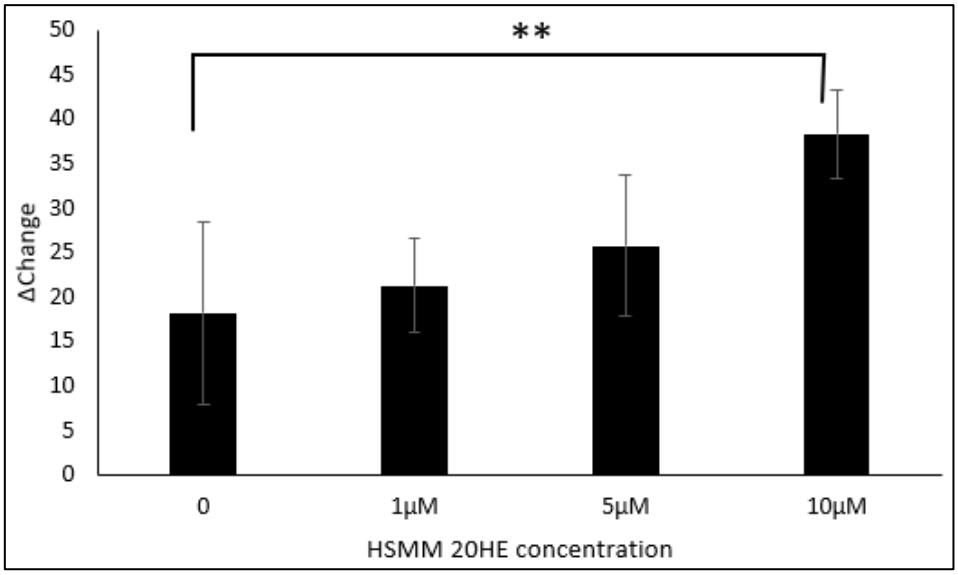


(Figure-41) Comparison of myoblasts growth dependent on media. DMEM (Left) [(a), (c), (e)] and SKGM recommended media (right) [(b), (d), (f)] were compared under light microscopy (Leica). Pictures were taken after a day (a) and (b), 4 days (c) and (d) and at the end of the experiment at 8 days after seeding (e) and (f).

Myoblasts confocal imaging

Results from four experiments each consisting of four coverslips treated with 0, 1, 5 & 10 µM 20HE overnight and investigated under confocal microscopy are summarised in (Figure-42). Similar results of those reported in preadipocytes were also observed in myoblasts. Significant increase (p=0.01) in the delta change in the 5 & 10 µM treated cells in comparison to the untreated and cells treated with 1µM 20HE. Delta change is the difference in signal intensity of the TMRE stain detected by the confocal microscopy system between the same field before and after adding FCCP. Increased signal intensity is indicative of increased polarity between the compartments of mitochondria and this membrane potential is harnessed for ATP synthesis. Adding FCCP allows the free transport of the protons between mitochondrial compartments diminishing polarity and signal intensity of the TMRE stain.

Mitochondria membrane potential investigation as an indicator of polarised active mitochondria revealed that 20HE has significant effects on mitochondrial respiration in both preadipocytes and myoblasts. Further application of these studies to mature adipocytes and differentiated skeletal muscle cells should be considered.



(Figure-42) Mitochondrial membrane potential differences after treatment with 20HE. A significant increase in the Δ change of recorded intensity after treatment with 10µM of 20HE in comparison to the vehicle treated cells (*p*=0.01) which means significant increase in mitochondrial function in response to higher doses of treatment with 20HE.

4.2 Molecular mechanisms- Omics approach

The suffix omics refers to comprehensive investigation. It is applied to genes (Genomics), proteins (Proteomics) or phenotypes (Phenomics) among other studies. After the targeted and specific effects of 20HE that were reported in this study, an omics approach was conducted investigating total effects of 20HE on the proteome (protein profile) and transcriptome (expressed genes profile). Protein and RNA extracted from the humanised SCID model treated with 20HE were used for the omics investigations.

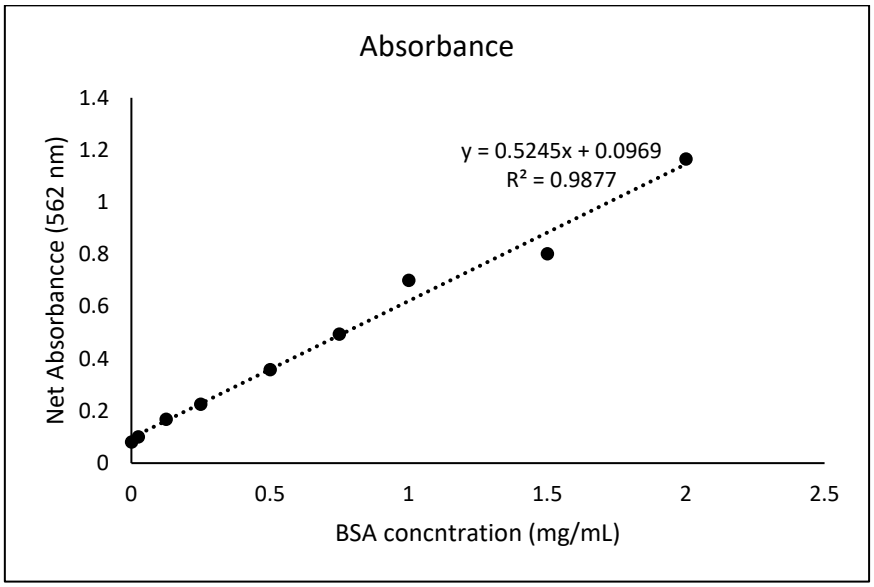
4.2.1 Proteomics

4.2.1.1 Protein extraction and concentration estimation

Proteins were extracted by homogenising the liver tissue in RIPA buffer and concentrations were estimated using BCA colorimetric assay (Figure-43). Proteins from all samples had concentrations that were too high, and the assay was not able to provide reliable estimation. Thus, protein samples were diluted twice, and concentration estimation was repeated after 1:500 dilution (Table-21).

4.2.1.2 Proteome analysis using the ELITE Orbitrap LC/MSMS

Data analysis was conducted on the raw files and .msf files created from Proteome Discoverer software. Scans from raw files were viewed using RawMeat V.2 where full scans represent parent peptides and MSMS scans show more peaks within the parent compound detected and the numbers of these scans for one sample consisting of 8 fragments as an example is reported in (Table-22). Trypsin/lysin digestion controls were studied for all samples as shown in (Figure-44).



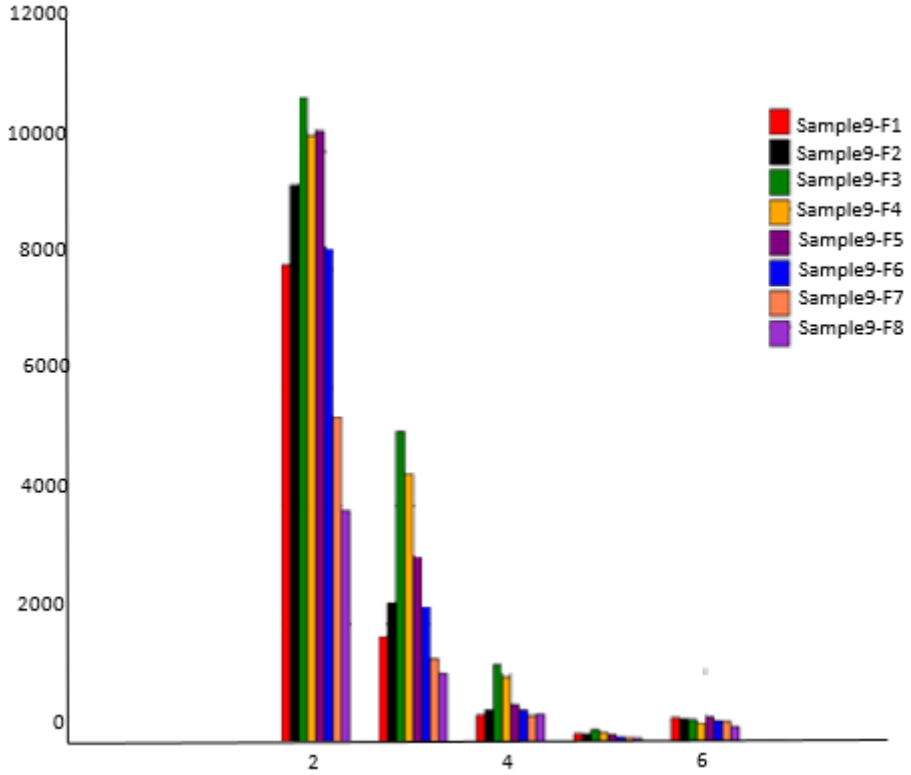
(Figure-43) Calibration curve of standards for the estimation of protein concentration. BSA was used as the standard and the range was up to 2.5 mg/ml.

Sample ID	Dilution into 0.85mg/mL in 20µl		Absorbance	Concentration estimation mg/mL
	Vol. from stock	**PBS (µL)**		
5	12	8	0.71	1.41
7	14.1	5.9	0.60	1.21
9	13.1	6.9	0.65	1.30
12	14.3	5.7	0.59	1.19
14	16.4	3.6	0.52	1.03
16	12.3	7.7	0.69	1.38
18	11.7	8.3	0.72	1.45
19	19.2	0.8	0.44	0.89
29	13.4	6.6	0.63	1.26
966	14.8	5.2	0.58	1.15

(Table-21) Results of protein concentration estimation was done using Pierce BCA protein assay kit. The absorbance was measured at 562nm at room temperature.

Raw file Sample9	Number of Full Scans	Number of MS2 Scans
Fragment 1	4359	10915
Fragment 2	3952	12900
Fragment 3	2836	18145
Fragment 4	3189	16433
Fragment 5	3618	14722
Fragment 6	4220	11741
Fragment 7	5518	7811
Fragment 8	6190	5922

(Table-22) Full Scans of parent peptides detected using the LCMS and MS2. Full scans present the number of parent peptides detected in the first MS scan and these were further analysed with a second MS giving rise to additional peptides (MS2 scans).



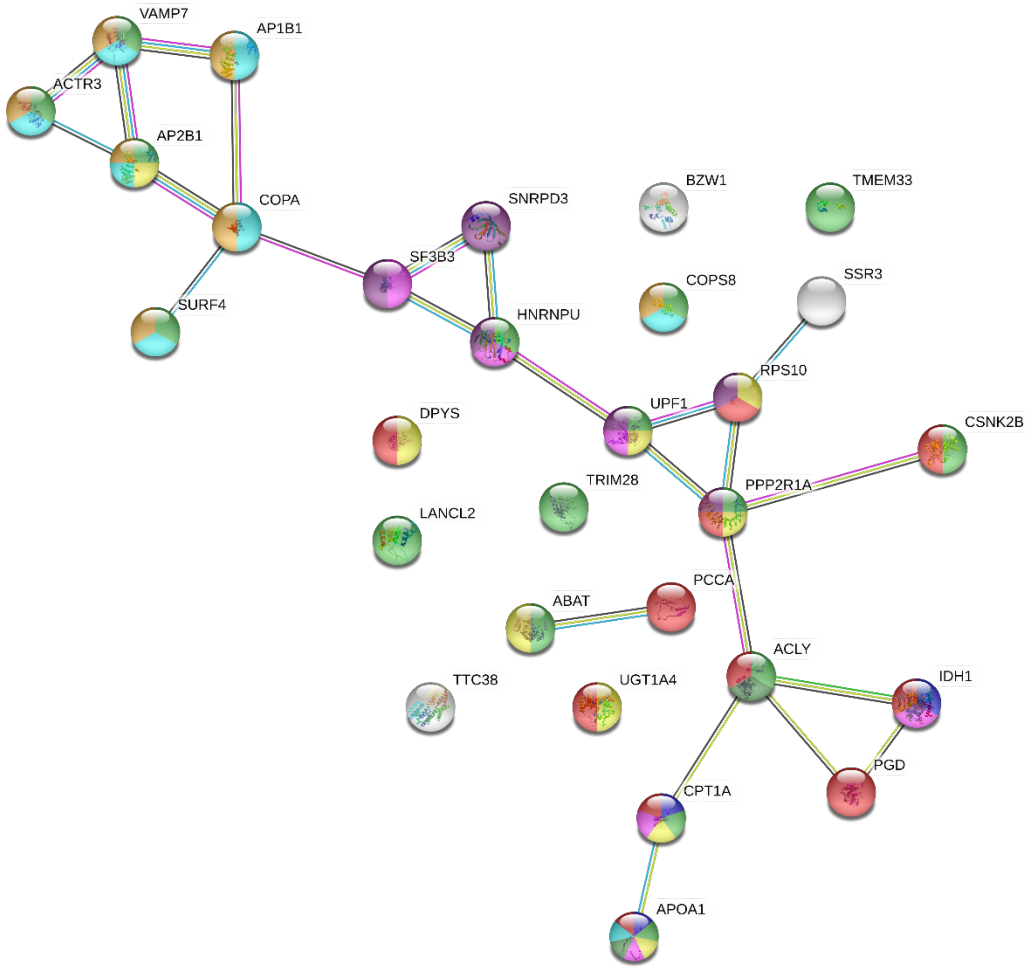
(Figure-44) Charge distribution of N-Terminus. For the digestion to have worked the charge of 2 is expected to indicate acceptable quality of digestion. Most of the peptides attained this quality**.** This graph presents the eight fragments of one sample.

4.2.1.3 Proteomics data analysis

Output files from proteome discoverer were analysed as detailed in methods using excel. 1664 proteins were quantified and detected in both 20HE and placebo group where significant differences in abundance were found in 32 proteins. 70 proteins were specific to the 20HE treated group whereas 68 proteins were specific to the vehicle treated group. Computational predictions based on the proteins that had *p*-value of less than 0.05 showed possible interactions between these proteins using STRING which is a web-based search tool identifying protein-protein interactions (91) (Figure-45).

The 70 proteins that were only detected in the 20HE group were also analysed on STRING and some of the enriched pathways are shown in (Figure-46). Metabolism and metabolism of proteins pathways were enriched after 20HE treatment according to the data from Gene Ontology and Reactome.

Looking at the 68 proteins uniquely detected in the placebo group one could cautiously presume possible suppression of these in response to 20HE. Utilising STRING, 54 of the 69 proteins were shown and enriched pathways are presented in (Figure-47). Though many of the enriched pathways in the placebo group are not unique to the group and also were found in the 20HE or the shared protein analyses, the proteins detected within the pathways differ. Which indicate the enrichment of alternate pathways in response to the treatment. For example, the Reactome metabolism pathway was enriched in all 3 analyses; shared proteins among the groups, proteins specific to 20HE and protein only detected in the placebo group (Figures-45,46 and 47). Proteins detected in the metabolism pathway in the first group included: RPS10, ACLY, PGD, DPYS, CSNK2B, PP2R1A, PCCA, UGT1A4, CPT1A and APOA1. While the 20HE group proteins within the same pathway included: GBE1, GPHN, RPL23A, RPS20, PON3, RAN, GSTO1, PSMC3, PAICS, ETFA, LDHB, AASS, DECR2 and CMPK1. Lastly, proteins detected within the pathway in the placebo group included: ACSM3, SULT1A3, CRYM, UQCRC2, ETFA, CMPK1, PRPS2, PAICS, LDHB, APRT, CPNE3, RPS9, PSMB8, GSTZ1 and LSS.



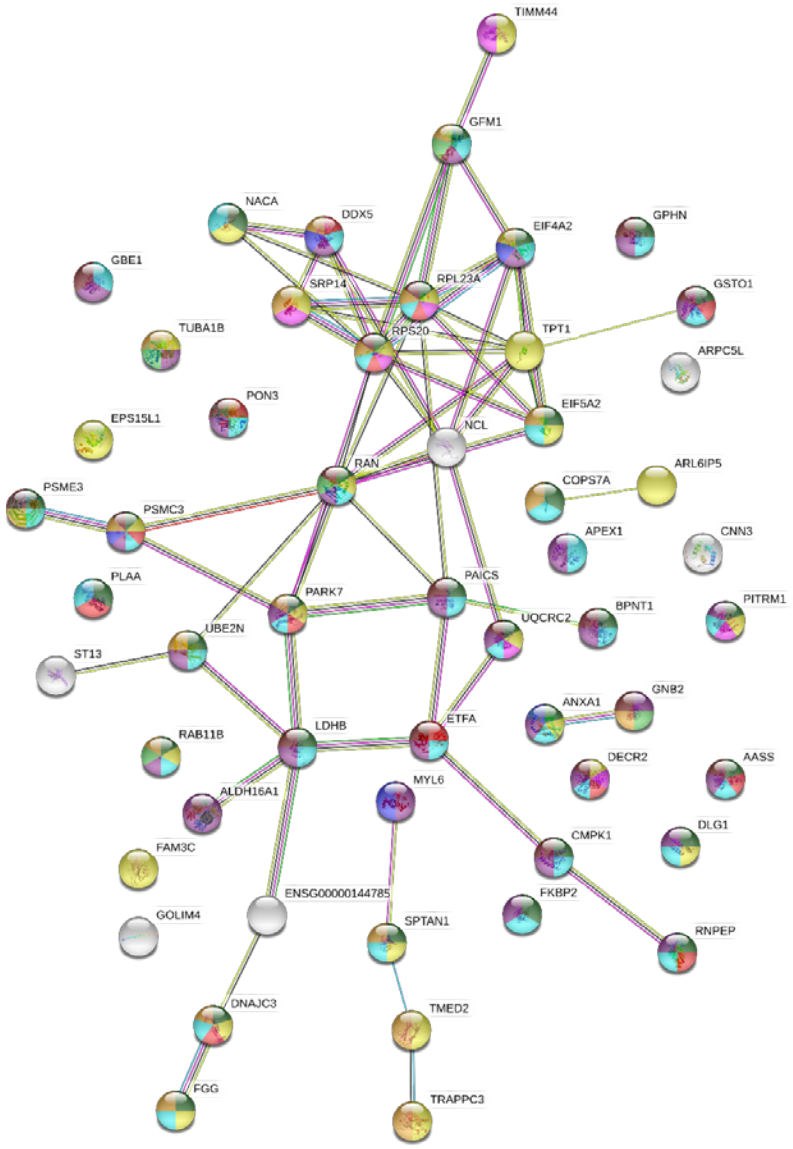
GO_biologial processes enriched pathways

- Regulation of lipid catabolic process
- Positive regulation of cellular process
- Cellular catabolic process
- Regulation of catabolic process
- Cholesterol biosynthetic process

Reactome enriched pathways

- Vesicle-mediated transport
- Membrane trafficking
- Metabolism
- Metabolism of RNA

(Figure-45) Protein-protein interaction network. The interactions between the identified 32 proteins that were significantly altered in abundance between the 20HE and placebo treated groups were generated using STRING. Colour codes within each node represents the protein involvement in the specified enrichment pathways from GO biological processes and Reactome (91).



GO-biologial processes enriched pathways

- Primary metabolic process
- Cellular catabolic process
- Transport
- Protein targeting
- Organonitrogen compound metabolic process

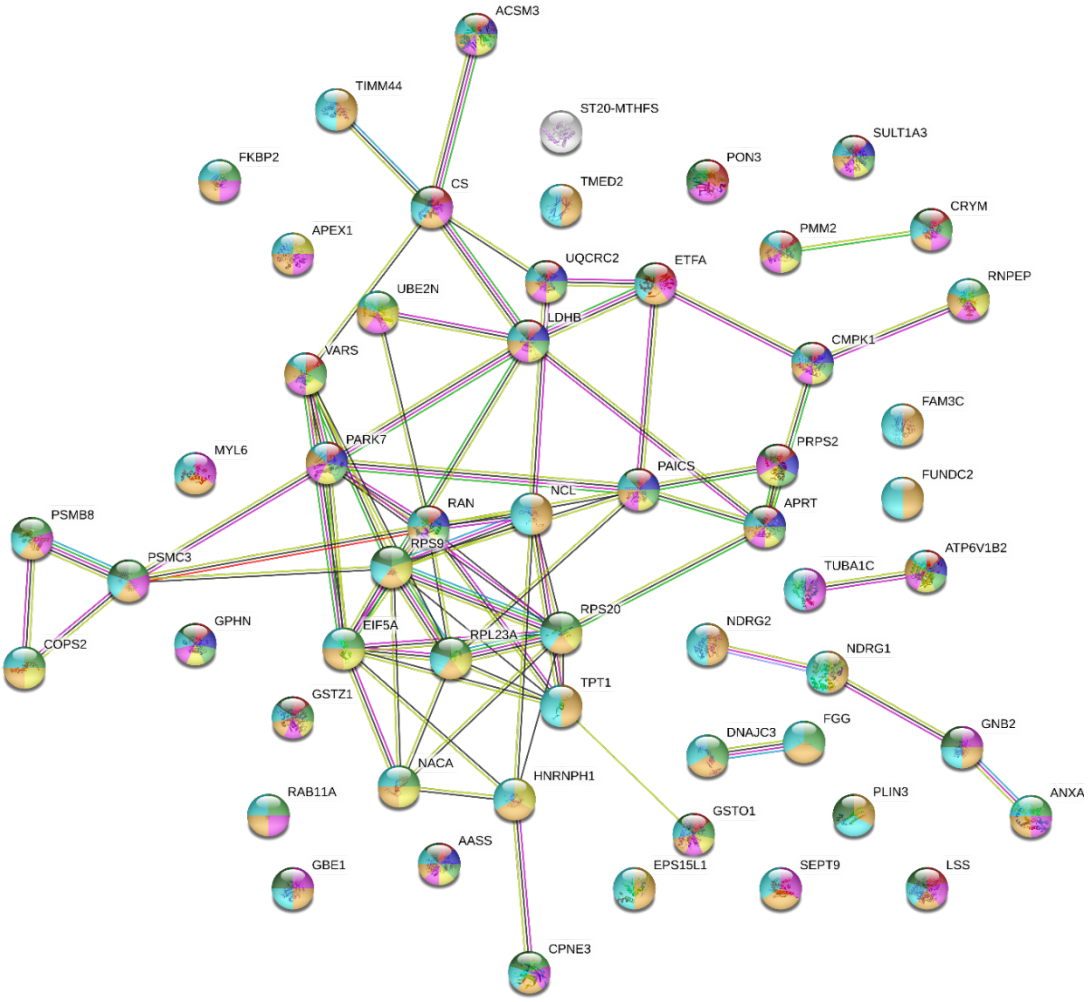
Reactome enriched pathways

- Metabolism of proteins
- Metabolism

GO-molecular mechanisms enriched pathways

- Catalytic activity
- GTPase activity
- ATPase activity coupled

(Figure-46) Specific proteins abundant in the 20HE treated group. Figure shows the interactions network according to enriched pathways against Gene ontology and Reactome databases. Colour codes within each node represents the protein involvement in the specified enrichment pathways from GO biological processes and molecular functions and Reactome (91).



GO-biologial processes enriched pathways

- Cellular nitrogen compound metabolic process
- Small molecule metabolic process
- Nucleotide metabolic process
- Organonitrogen compound metabolic process

GO-cellular component enriched pathways

- Cytoplasmic part
- Cytoplasm

GO-molecular mechanisms enriched pathways

- Catalytic activity

Reactome enriched pathways

- Metabolism

(**Figure-47) Enriched pathways according to input proteins specific to placebo group.** Proteins list was analysed using STRING against Gene Ontology (GO) and Reactome databases (91). Colour codes within each node represents the protein involvement in the specified enrichment pathways.

4.2.2 Transcriptome analysis

The second omics approach utilised was transcriptome analysis where extracted RNA from the humanised SCID model treated with 20HE or placebo was used.

4.2.2.1 RNA sample preparation

Results of quality check using the NanoDrop after *in-vitro* transcription and fragmentation of all samples in triplicates are summarised in (Table-23). Since the quality of the input RNA was good all downstream reactions yielded sufficient amounts which were quantified as per manufacturer's recommendations after the overnight *in-vitro* transcription (IVT) and single stranded cDNA synthesis.

4.2.2.2 Results from in-house Data Analysis

Analysis was conducted using TAC 4.0 software and showed 37 differentially expressed genes (Table-24) when comparing 20HE treated group with vehicle treated group which is illustrated in (Figure-48). Expression analysis were set at fold change < -2 or > 2 and *p*-value≤ 0.05 was considered significant at gene level. Of these 37 genes, HES1 gene which is a transcription factor that prevents transcription was significantly downregulated with *p*-value of 2.27E-05, it is a negative regulator if myogenesis adding to the data indicating increased muscle mass in response to 20HE treatment. This data, though interesting, it comes from a compromised mice model and reliability on this is restricted.

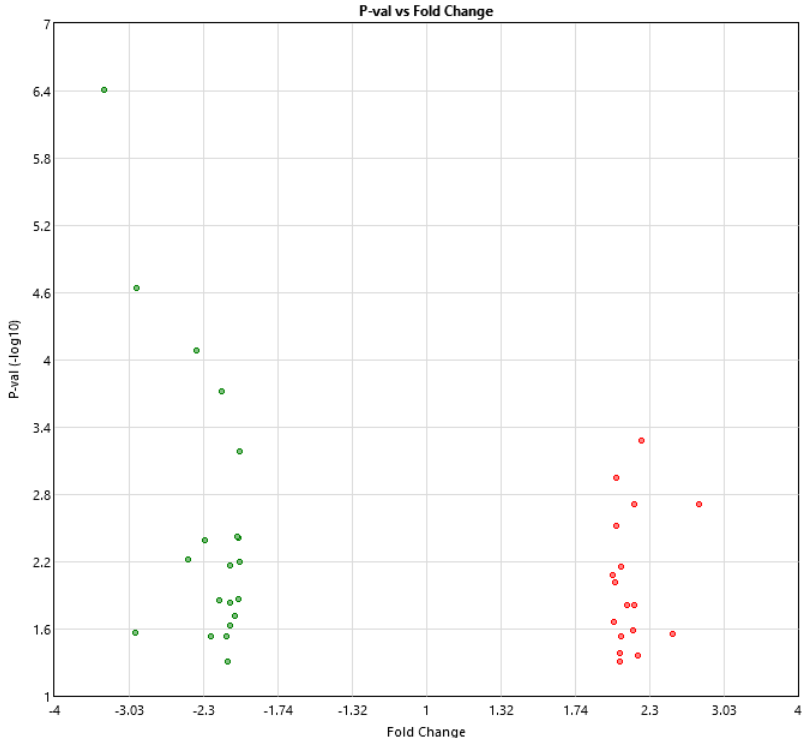
Sample	Well position	cRNA Yield after IVT (µg)	Amount required (µg)	cDNA Yield after 2nd cycle ss-cDNA synthesis (µg)	Amount required (µg)
5	A1	1784.9	15	490.0	5.5
	A2	1301.4	15	490.0	5.5
	A3	1431.8	15	390.0	5.5
7	A4	1194.8	15	398.4	5.5
	A5	1239.6	15	63.6	5.5
	A6	1172.9	15	403.8	5.5
9	A7	484.5	15	234.7	5.5
	A8	1128.2	15	400.4	5.5
	A9	1359	15	397.1	5.5
12	A10	777.1	15	414.3	5.5
	A11	668.2	15	352.8	5.5
	A12	711.5	15	347.9	5.5
14	B1	1045.6	15	738.3	5.5
	B2	741.8	15	356.2	5.5
	B3	1396.8	15	391.5	5.5
16	B4	646.9	15	377	5.5
	B5	760.1	15	341.4	5.5
	B6	641	15	350.8	5.5
18	B7	1282.8	15	434.5	5.5
	B8	1163.4	15	403.3	5.5
	B9	459.2	15	164.3	5.5
19	B10	920.3	15	389.2	5.5
	B11	691.6	15	384.5	5.5
	B12	651.7	15	356.7	5.5
29	C1	1338.6	15	488.4	5.5
	C2	788.5	15	431.2	5.5
	C3	1099.6	15	410.2	5.5
966	C4	1080.6	15	430.3	5.5
	C5	1224.6	15	406.9	5.5
	C6	1181.5	15	437.6	5.5

(Table-23) Quality check of cRNA & cDNA concentrations. Samples were measured using NanoDrop during the preparation of RNA samples for transcriptome analysis.

Gene Symbol	20HE Avg (log2)	Vehicle Avg (log2)	Fold Change	*p*-val
WNK1	4.65	3.61	2.06	0.05
STK36	3.66	4.73	-2.1	0.05
G6PC	13.4	12.26	2.2	0.04
ODF2L	5.39	4.35	2.06	0.04
DIP2C	4.37	5.45	-2.11	0.03
MRPL42	7.27	8.42	-2.23	0.03
NAT6	5.6	4.56	2.07	0.03
PPM1D	6.38	5.06	2.5	0.03
HSPA1B; HSPA1A	10.14	11.71	-2.96	0.03
DUSP1	10.08	8.97	2.16	0.03
RANBP1	6.9	7.96	-2.08	0.02
GATS	5.51	4.51	2.01	0.02
CXXC1	3.94	4.97	-2.05	0.02
HPS5	9.26	8.14	2.17	0.02
MYSM1	6.99	5.92	2.11	0.02
GMNC	4.48	5.54	-2.08	0.01
REPS2	5.51	6.63	-2.17	0.01
MANF	4.59	5.61	-2.02	0.01
MT1L	15.21	14.2	2.02	0.01
CD180	4.27	3.26	2	0.01
POGZ	5.32	4.28	2.06	0.01
MYO5A	3.85	4.91	-2.08	0.01
BLZF1	6.09	7.1	-2.01	0.01
BBX	5.74	7.02	-2.43	0.01
PGD	4.79	5.98	-2.28	<0.01
MCM4	5.68	6.7	-2.02	<0.01
KARS	8.41	9.43	-2.02	<0.01
ZNF573	5.29	4.27	2.02	<0.01
PTPRO	3.87	2.4	2.76	<0.01
INCA1	5.02	3.91	2.16	<0.01
HNRNPCL3	4.3	3.28	2.03	<0.01
CDC42EP4	4.31	5.32	-2.01	<0.01

ZDHHC18	7.31	6.16	2.22	<0.01
ZNF420	4.6	5.71	-2.15	<0.01
STIP1	7.4	8.64	-2.36	<0.01
HES1	6.68	8.24	-2.95	<0.01
HERPUD1	10.51	12.24	-3.32	<0.01
G6PC	13.4	12.26	2.2	0.0434
MRPL42	7.27	8.42	-2.23	0.0294
HPS5	9.26	8.14	2.17	0.0155
MANF	4.59	5.61	-2.02	0.0136
CDC42EP4	4.31	5.32	-2.01	<0.01
ZNF420	4.6	5.71	-2.15	<0.01
HES1	6.68	8.24	-2.95	2.27E-05
HERPUD1	10.51	12.24	-3.32	3.85E-07

(Table-24) Differentially expressed genes between 20HE and vehicle treated groups. Data were created using TAC 4.0 software. Many of these genes express key functions such as the G6PC gene expresses a key enzyme for glucose homeostasis, HES1which is a negative regulator of myogenesis and MRPL42 that plays a role in organelle biogenesis and maintenance.



(Figure-48) Volcano plot of altered genes after 20HE treatment. Number of differentially expressed genes; 17 were upregulated in the 20HE treated group while 20 were downregulated in comparison to vehicle treated group.

Gene table without any filters applied, showing all genes detected from the transcriptome array was exported and 230 human liver drug metabolism genes were investigated. Of these 230 genes only 9 were found to be significant with *p*-value ≥0.05 summarised in (Table-25). In addition, 104 mitochondria related genes were investigated and only three were found to be significant while other few showed no significance in differential expression in response to 20HE treatment, also summarised in (Table-25).

	20HE Avg (log2)	Vehicle Avg (log2)	Fold Change	*p*-val	Gene Symbol	Description
Phase1	3.88	4.64	-1.69	0.0411	CYP17A1	cytochrome P450, family 17, subfamily A, polypeptide 1
	8.62	9.62	-1.99	0.0545	CYP1A1	cytochrome P450, family 1, subfamily A, polypeptide 1
	6.25	5.86	1.32	0.0227	GZMB	granzyme B
	4.59	3.96	1.54	0.0578	ALDH1A2	aldehyde dehydrogenase 1 family, member A2
	8.92	9.32	-1.32	0.0432	ALDH5A1	aldehyde dehydrogenase 5 family, member A1
Phase2	5.12	4.61	1.43	0.0251	IFT22	intraflagellar transport 22
	3.25	3.7	-1.37	0.0251	GAD2	glutamate decarboxylase 2
	2.39	2.82	-1.35	0.0278	LPO	lactoperoxidase
	3.04	2.58	1.38	0.0407	NOS3	nitric oxide synthase 3 (endothelial cell)
	5.58	5.16	1.34	0.0257	NQO1	NAD(P)H dehydrogenase, quinone 1
	7.69	7.14	1.46	0.0339	GALNT1	polypeptide N-acetylgalactosaminyltransferase 1
Mitochondria Energy Metabolism	4.22	3.62	1.51	0.0256	ATP4A	ATPase, H+/K+ exchanging, alpha polypeptide
	8.85	9.16	-1.24	0.0166	NDUFB8	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 8, 19kDa
	7.27	8.42	-2.23	0.0294	MRPL42	mitochondrial ribosomal protein L42

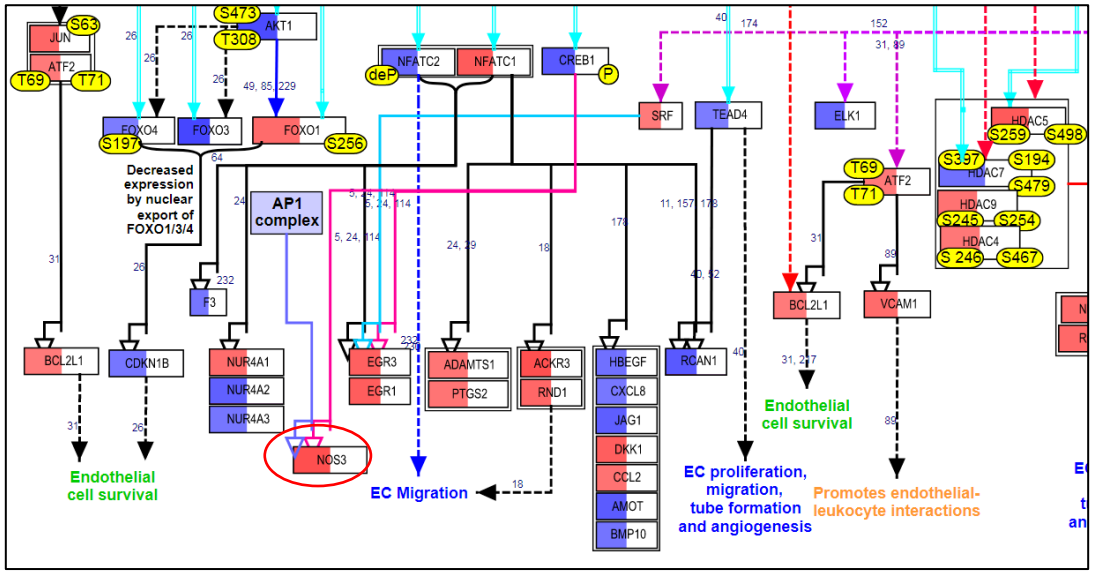
(Table-25) Differentially expressed genes categorised according the proteins expressed. Enzymes involved in Phase I and phase II drug metabolism processes and Mitochondria energy and metabolism are shown.

4.2.3 Functional Enrichment analysis (pathway analysis)

Datasets obtained from different omics approaches are generally large and overwhelming. An important tool to interpret such data is pathway analysis (PA), also known as functional enrichment analysis. Instead of looking at these datasets -or in this study gene lists- as a large number of genes in isolation of their biological context, pathway analysis allows easier detection of related genes that can be differentially expressed in treatment group when compared to control (89). Biological causes are more easily explored when examining gene expression within these pathways.

4.2.3.1 Transcriptome

Initial PA was conducted using the TAC 4.0 software. The most significant hit found was the VEGFA-VEGFR signalling pathways (Figure-49). This in part could be due to the significant FDR *p*-value of the NOS3 gene.

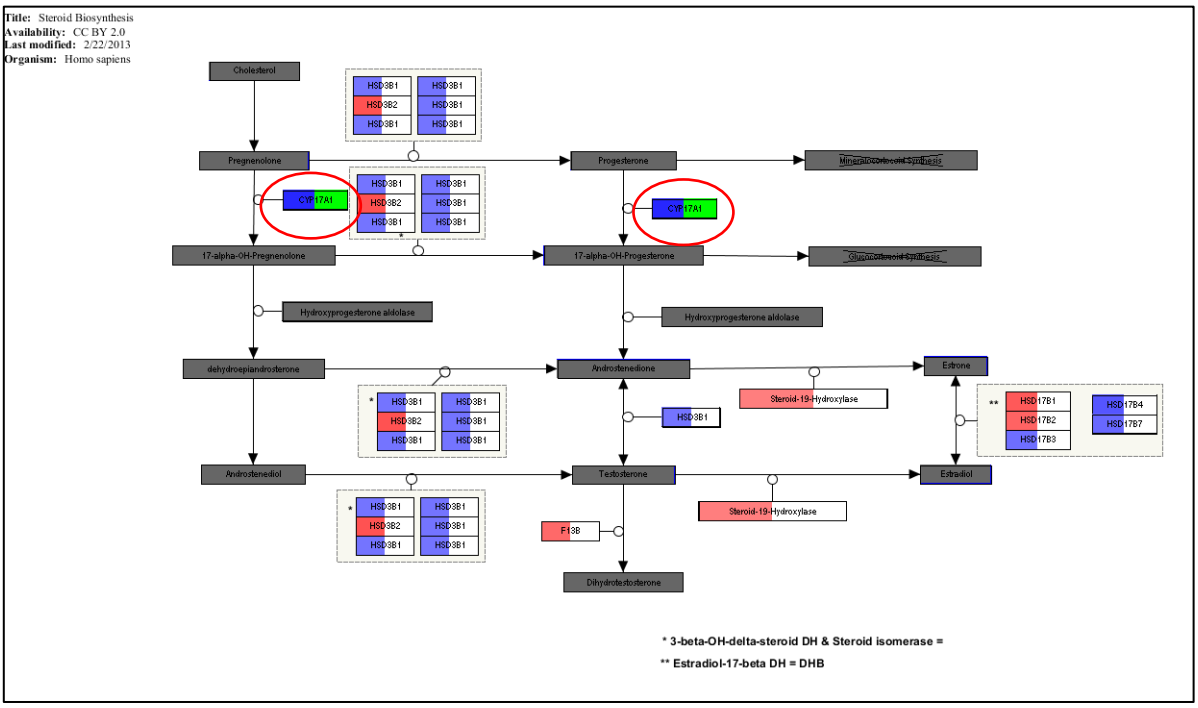


(Figure-49) Effect of 20HE on the expression of the VEGFA-VEGFR signalling pathway components. NOS3 (circled) was reported as significant in the transcriptome data. The red and blue colours in the genes represent over and under representation of the gene respectively in the treatment group compared to control. Pathway analysis conducted using TAC 4.0 software.

Further PA analysis was done in collaboration with Qatar Computational Research Institute and exported gene list was looked at against KEGG pathways. Many interesting pathways revealed significance. These included steroidogenesis, hypertrophy\anabolic, mitochondrial and ER stress pathways. These pathways were briefly explored in the following section.

Steroidogenesis pathway

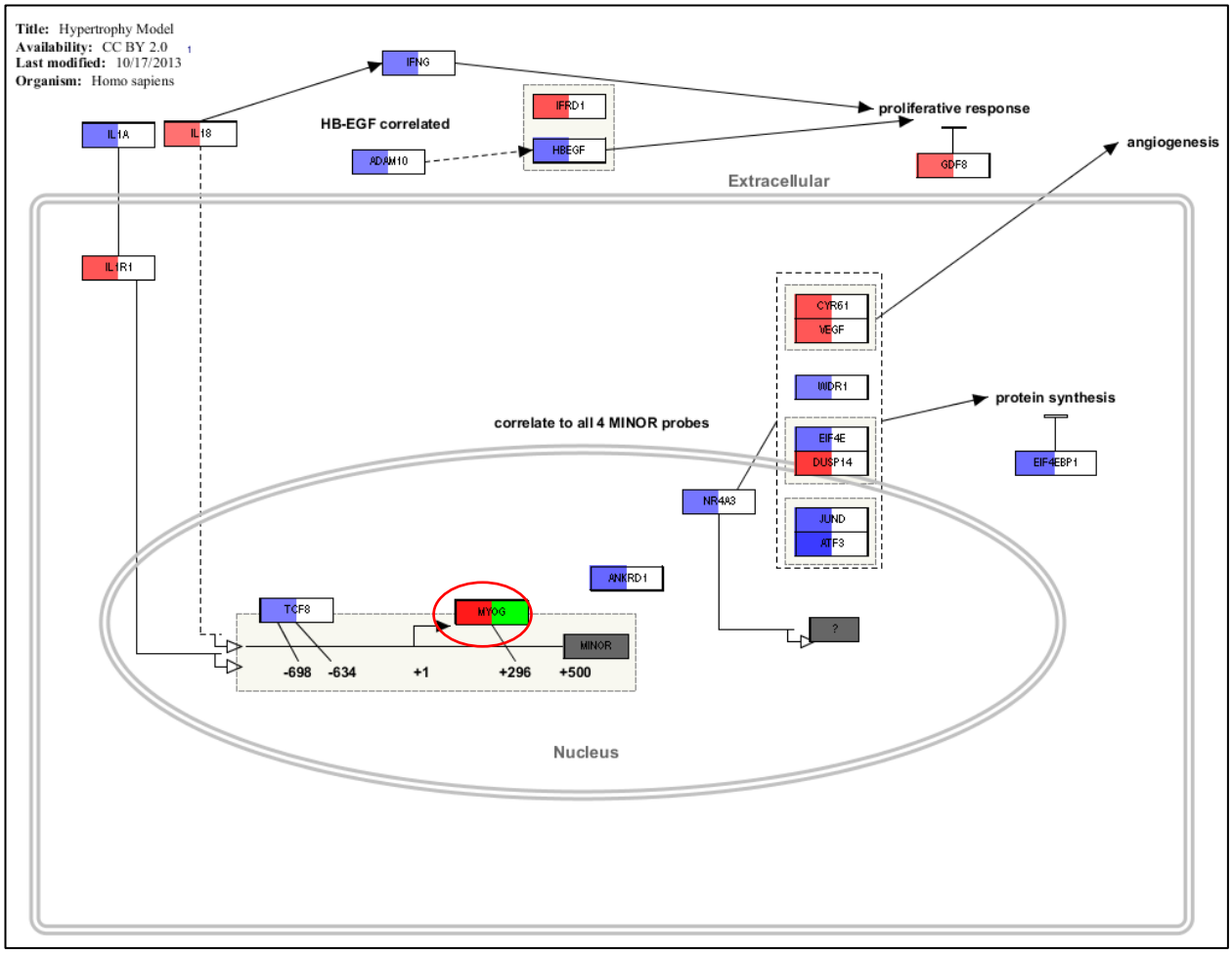
Pathway analysis showed that steroidogenesis pathway was significant with *p*-value=0.01 and one of the genes within this pathway that was found significantly downregulated was CYP17A1 in the 20HE treated group compared to the placebo group (Figure-50). It Plays an important role in steroidogenesis and it also controls the levels of mineralocorticoids which influence blood pressure, glucocorticoids involved in immune and stress responses, and androgens and oestrogens.



(Figure-50) Steroidogenesis pathway. CYP17A1 (circled) gene was significantly downregulated in the 20HE treated cohort and this downregulation affected downstream reactions. The red and blue colours in the genes represent over and under representation of the gene respectively in the treatment group compared to control. Pathway analysis conducted using TAC 4.0 software

Hypertrophy/anabolic pathway

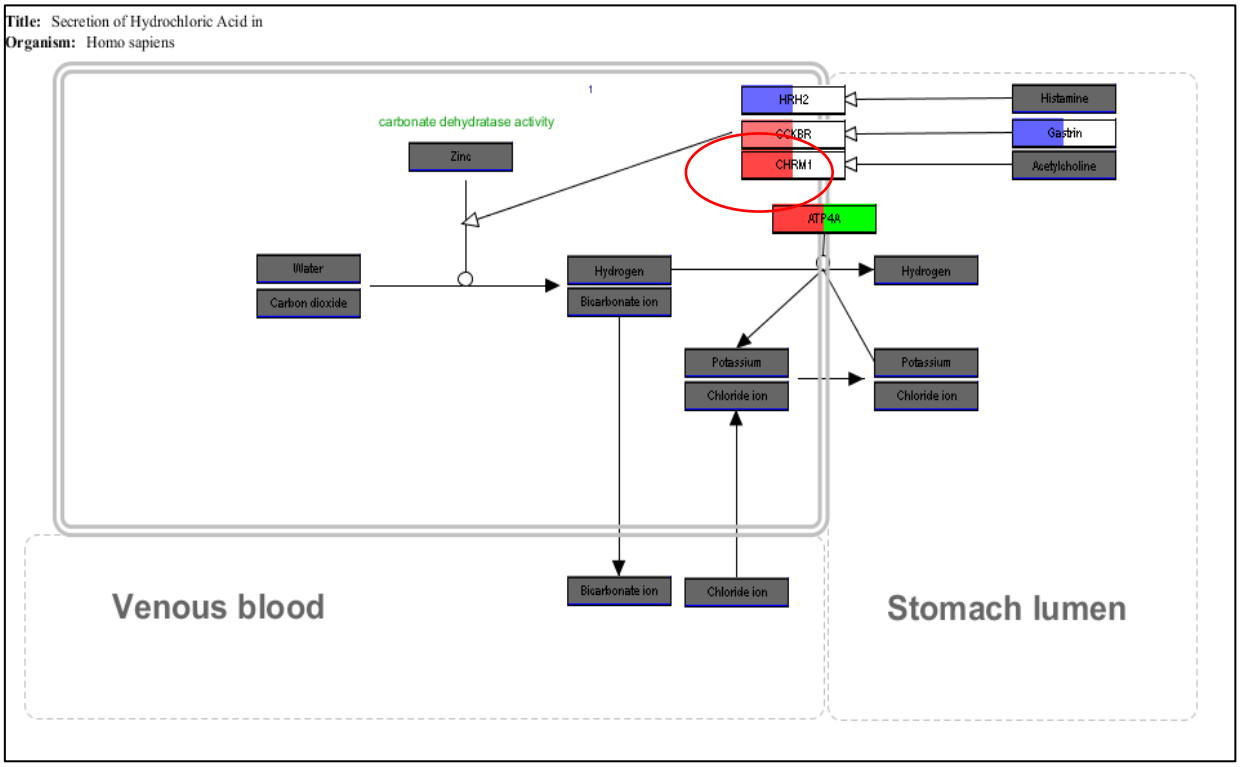
Another significant pathway reported was the anabolic pathway and according to the data from the transcriptome analysis the gene Myogenin (MYOG) which is a transcriptional activator that functions in promoting the transcription of genes that are muscle-specific taking part in the hypertrophy/anabolic pathway and it was significantly downregulated (Figure-51). This is in line with reports of increased muscles mass in response to ecdysteroid treatment.



(Figure-51) Hypertrophy/Anabolic pathway showing MYOG gene (circled) that was significantly downregulated in response of 20HE treatment indicated by the red and green colour respectively in the PA analysis. The red and blue colours in the genes represent over and under representation of the gene respectively in the treatment group compared to control. Pathway analysis conducted using TAC 4.0 software

Mitochondria related pathway

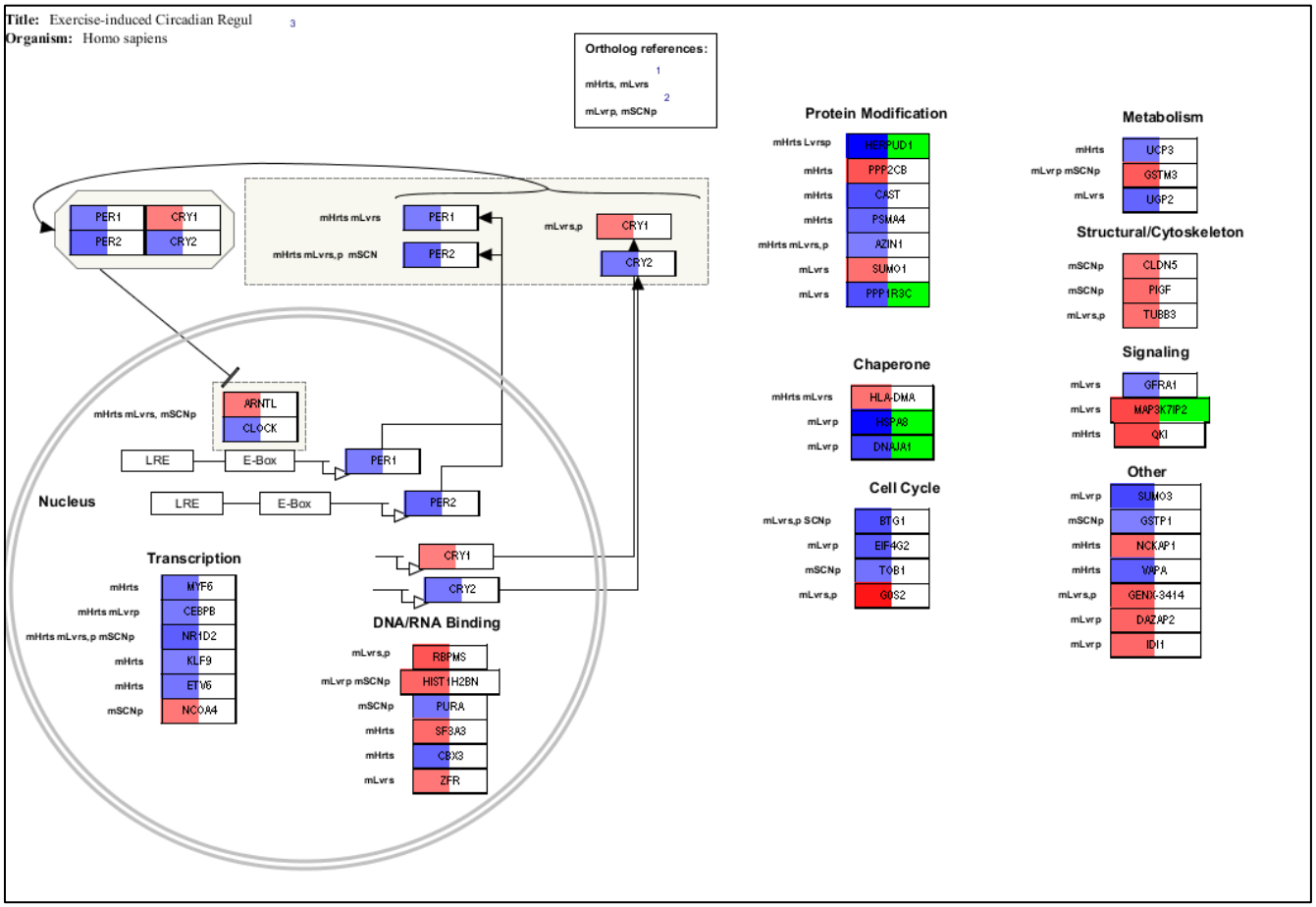
In the mitochondria related pathway, it was found that ATP4A (ATPase H+/K+ Transporting Subunit Alpha) proton pump was significantly downregulated in response to 20HE treatment (Figure-52). This enzyme catalyses ATP hydrolysis coupled with hydrogen and potassium ions across plasma membrane it aids in maintenance of acidic environment in the stomach.



(**Figure-52) Genetic pathway which relates to the mitochondrial function where ATP4A (circled) was significantly downregulated.** It harnesses the energy from ATP hydrolysis for the transport of H+ and K+ ion against their concentration's gradient. The red and blue colours in the genes represent over and under representation of the gene respectively in the treatment group compared to control. Pathway analysis conducted using TAC 4.0 software

Endoplasmic reticulum stress pathway

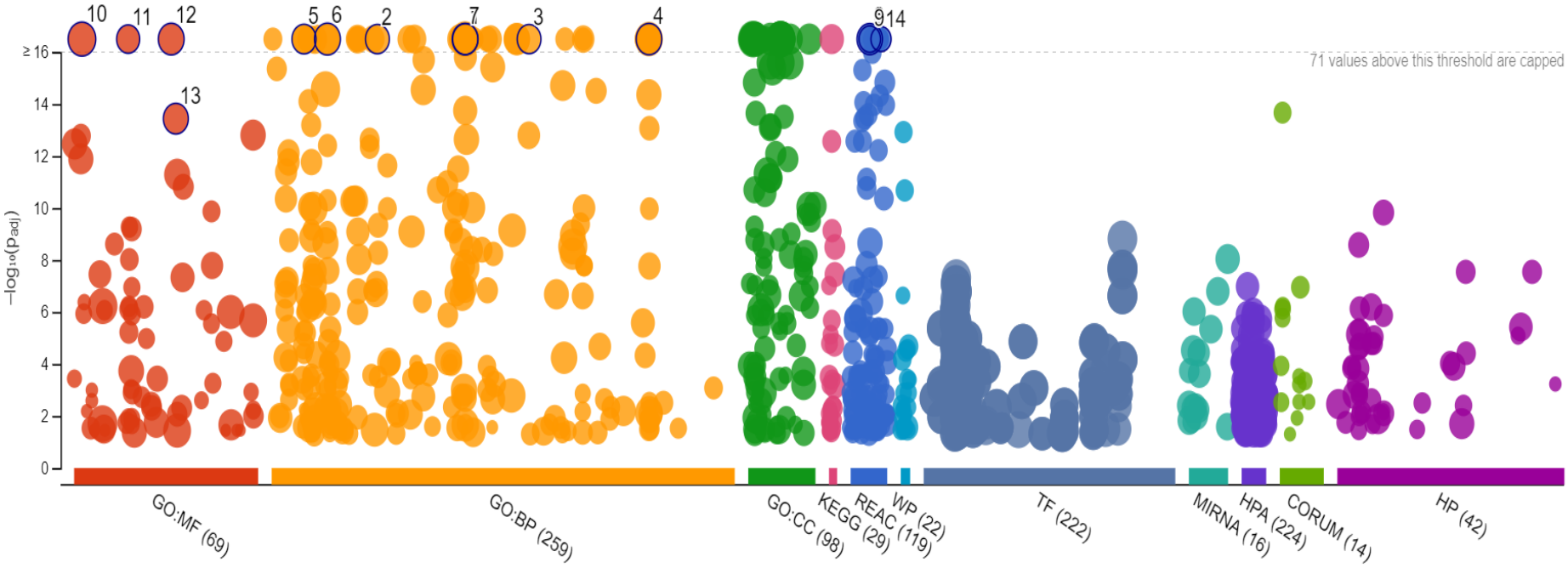
One of the most repeatedly found genetic functions from the transcriptome data was relating to Endoplasmic Reticulum stress. This was reported in the ER stress pathway (Figure-53) where many genes within the pathway were significantly differentially expressed including HERPUD1 which plays a role in responses to stress induced by misfolded proteins. Heat-shock cognate protein expressed by HSPA8 was also significantly upregulated and was reportedly functions in facilitating correct folding of proteins.



(Figure-53) ER stress pathway where many significantly differentially expressed genes were found including HERPUD1, PPP1R3C, HSPA8, DNAJA1 and MAP3K7IP2 (circled). The red and blue colours in the genes represent over and under representation of the gene respectively in the treatment group compared to control. Pathway analysis conducted using TAC 4.0 software

4.2.3.2 Combined functional enrichment analysis on the proteome and transcriptome data

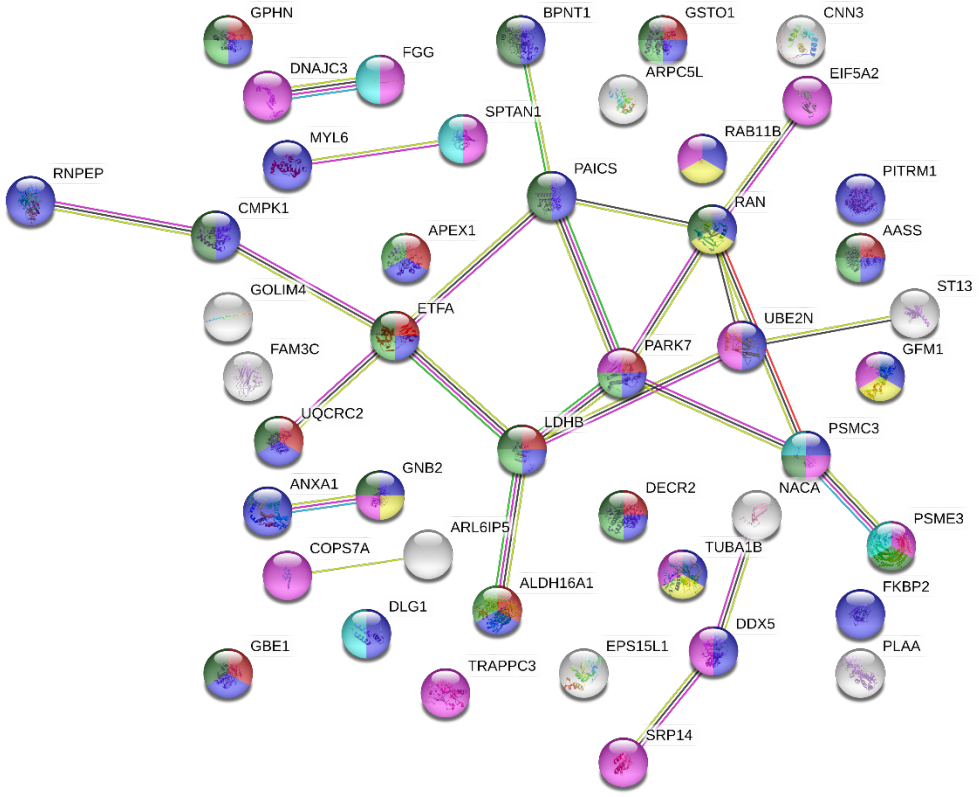
Protein gene IDs were obtained using uniport.org database converting protein accession number to their respective gene names. From the transcriptome data, significantly differentially expressed genes were compared to the protein list that is shared between the 20HE and placebo treated groups. Protein accession numbers, which are unique identifiers, were converted to their respective genes using uniport.org database (93) and then compared to the transcriptome data. 714 genes were matched from the transcriptome (significantly differentially expressed among 20HE vs. placebo) and proteome analysis. These were analysed using gProfiler -another web-based functional enrichment analysis tool, also known as over-representation analysis- showing possible enriched pathways and physiological functions based on the input gene list (92). Enriched pathways were identified with significant adjusted *p*-values (Figure-54). Metabolism, oxidation reduction process and other metabolic processes pathways were observed with significance like the results from the STRING functional protein networks (91).



(Figure-54) Functional enrichment analysis was conducted using gProfiler on gene list comprised of genes that were significantly altered in expression according to the transcriptome data and matched with their respective proteins according to the proteome data. X-axis shows the databases the gene list was ran against and the y-axis shows the adjusted *p*-value. Detailed interactions are presented in (appendix-5).

In addition, proteins that were only detected in the 20HE or vehicle treated group were converted to their gene names using uniport database and then the resulted gene lists were compared to the significantly over or under expressed genes from the transcriptome data. Out of the 70 proteins only detected in the 20HE treated group, 56 were also detected in the transcriptome with significance (Figure-55). The gene list consisting of these 56 genes were then analysed on STRING for the functional enrichment analysis investigation. The same was done for the vehicle treated group which yielded 61 matched proteins to the transcriptome data (Figure-56).

Additional analysis was conducted using FunRich version 3.1.3 which is a functional enrichment analysis software available online. An over view of some of the protein/gene lists created and used in the analysis are presented in (Figure-57) where the analysis was conducted using gene names from the transcriptome and protein accession numbers were converted to their respective gene names using UniProtKB (93). Interestingly, after converting proteins that were uniquely detected in either the placebo or 20HE group to their gene names it was shown that 36 of proteins uniquely detected in either group originated from same genes (Table-26). Which may suggest post transcriptional modification such as alternative splicing affecting the protein product.



GO-biologial processes enriched pathways

- Oxidation-reduction process

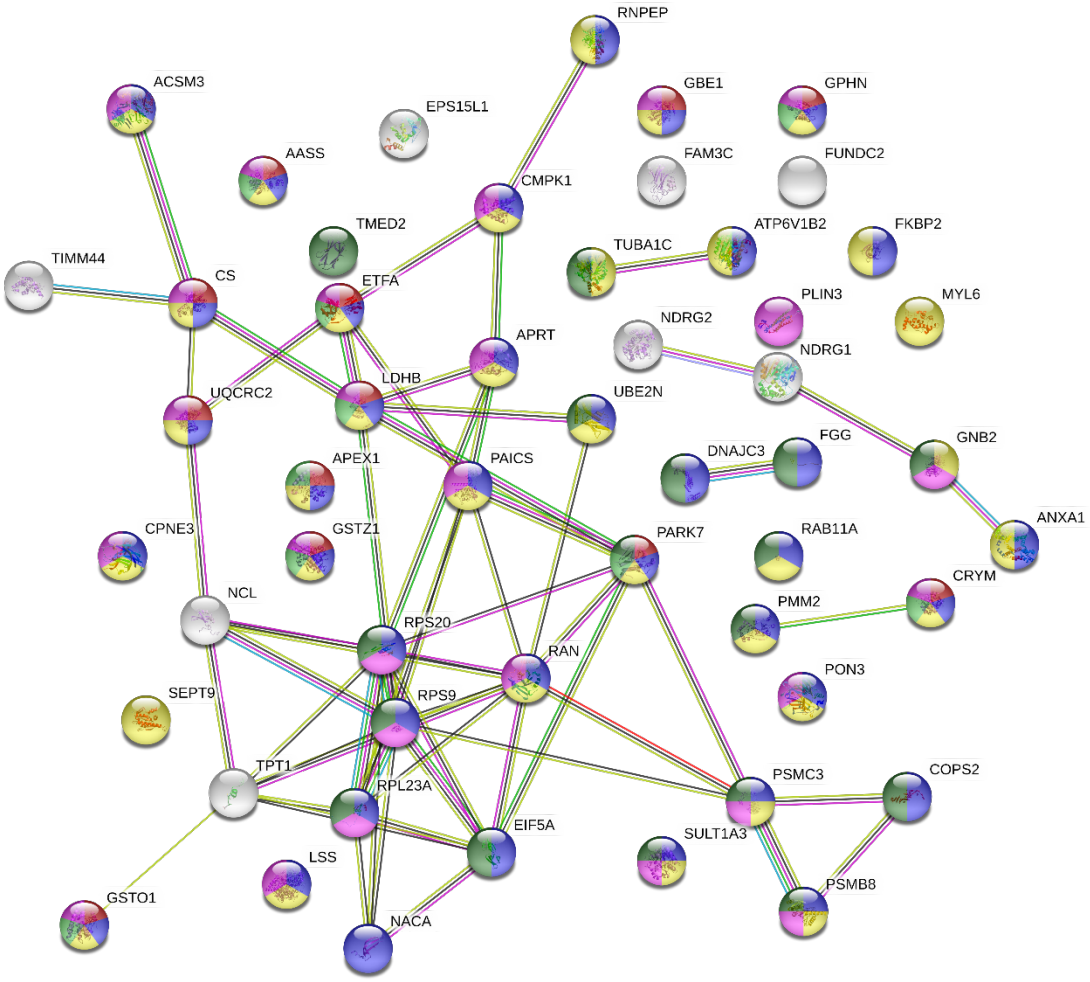
GO-molecular mechanisms enriched pathways

- Catalytic activity
- Oxidoreductase activity
- GTPase activity

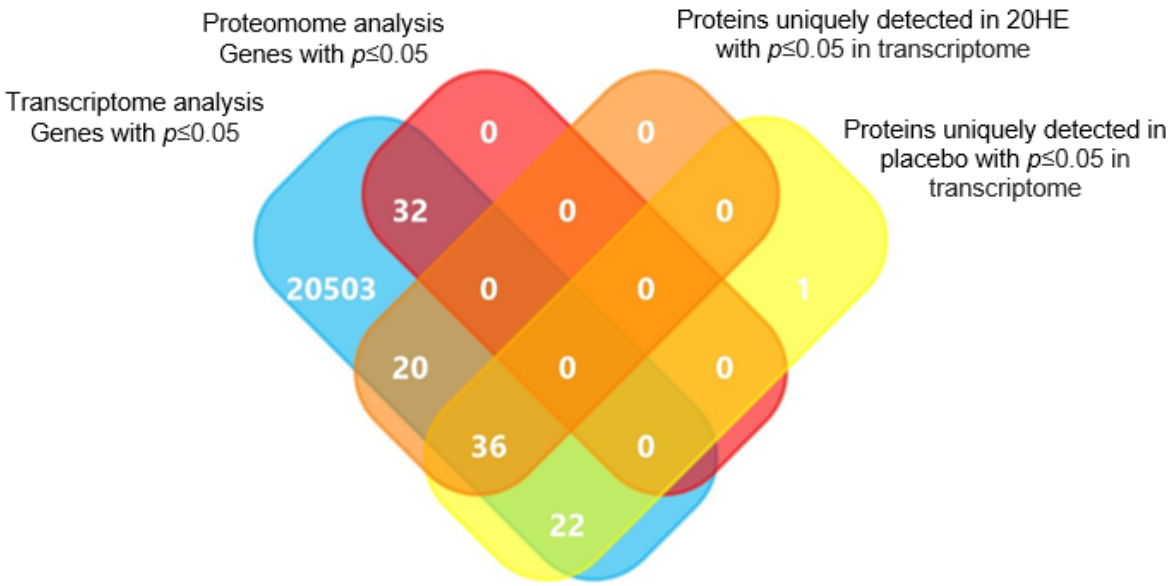
Reactome enriched pathways

- Metabolism of proteins
- Metabolism
- MAPK1/MAPK3 signalling

(Figure-55) Functional enrichment analysis of proteins unique to the 20HE-treated group matched with significantly differentially expressed genes detected in the transcriptome data. Analysis and figure were generated using STRING tool (91).



(Figure-56) Functional enrichment analysis of proteins unique to the vehicle-treated group matched with significantly differentially expressed genes detected in the transcriptome data. Analysis and figure was generated using STRING tool (91).

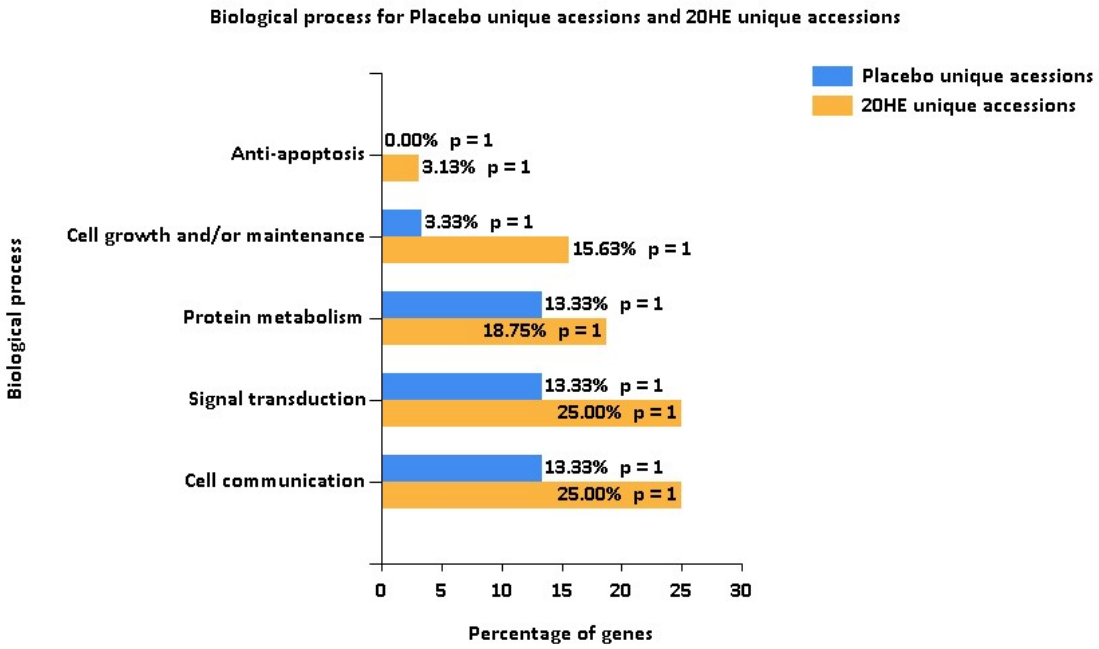


(Figure-57) Venn diagram of gene lists exported from the transcriptome and proteome analyses and uploaded to FuRich software. Protein accession numbers uniquely detected in either 20HE(Orange) or placebo (yellow) group were converted to their gene names and compared to significantly differentially expressed genes detected in the transcriptome (blue) and proteins that were significantly changed in abundance based on the proteomics data (red). Noteworthy that 36 genes expressed different proteins after treatment with 20HE compared to placebo.

	Gene name	expressed protein in 20HE	Ensemble.org transcript name and base pairs	expressed protein in Placebo	Ensemble.org transcript name and base pairs
1	SPTAN1	Q13813	Name: SPTAN1-202 Bp: 7889	A0A0D9SF54	Name: SPTAN1-222 Bp: 7812
2	ETFA	H0YLU7	Name: ETFA-215 Bp:917	P13804	Name: ETFA-202 Bp: 1346
3	GBE1	E9PGM4	Name: GBE1-205 Bp: 2513	Q04446	Name: GBE1-201 Bp: 2941
4	PAICS	E9PBS1	Name: PAICS-006 Bp: 1565	P22234	Name: PAICS-201 Bp: 3339
5	CMPK1	Q5T0D2	Name: CMPK1-004 Bp:1212	P30085	Name: CMPK1-005 Bp:950
6	PARK7	Q99497	Name: PARK7-001 Bp:1088	K7ELW0	Name: PARK7-003 Bp:795
7	APEX1	G3V5Q1	Name: APEX1-016 Bp:952	P27695	Name: APEX1-013 Bp: 671
8	RPS9	B5MCT8	Name: RPS9-007 Bp:944	P46781	Name: RPS9-001 Bp:772
9	ATP6V1B2	H0YC04	Name: Not-found Bp:	P21281	Name: ATP6V1B2-001 Bp:2865
10	GNB2	C9JIS1	Name: GNB2-010 Bp:910	P62879	Name: GNB2-006 Bp:1641
11	GSTO1	Q5TA02	Name: GSTO1-004 Bp:829	Q5TA01	Name: GSTO1-201 Bp: 900
12	MYL6	F8VPF3	Name: MYL6-214 Bp: 411	G8JLA2	Name: MYL6-201 Bp:664
13	GSTZ1	A0A0A0MR33	Name: Not-found Bp:	G3V5T0	Name: GSTZ1-007 Bp: 795
14	LDHB	A8MW50	Name: LDHB-004 Bp:744	P07195	Name: LDHB-002 Bp: 1538
15	RPL23A	P62750	Name: RPL23A-001 Bp:1562	K7EMA7	Name: RPL23A-006 Bp: 639

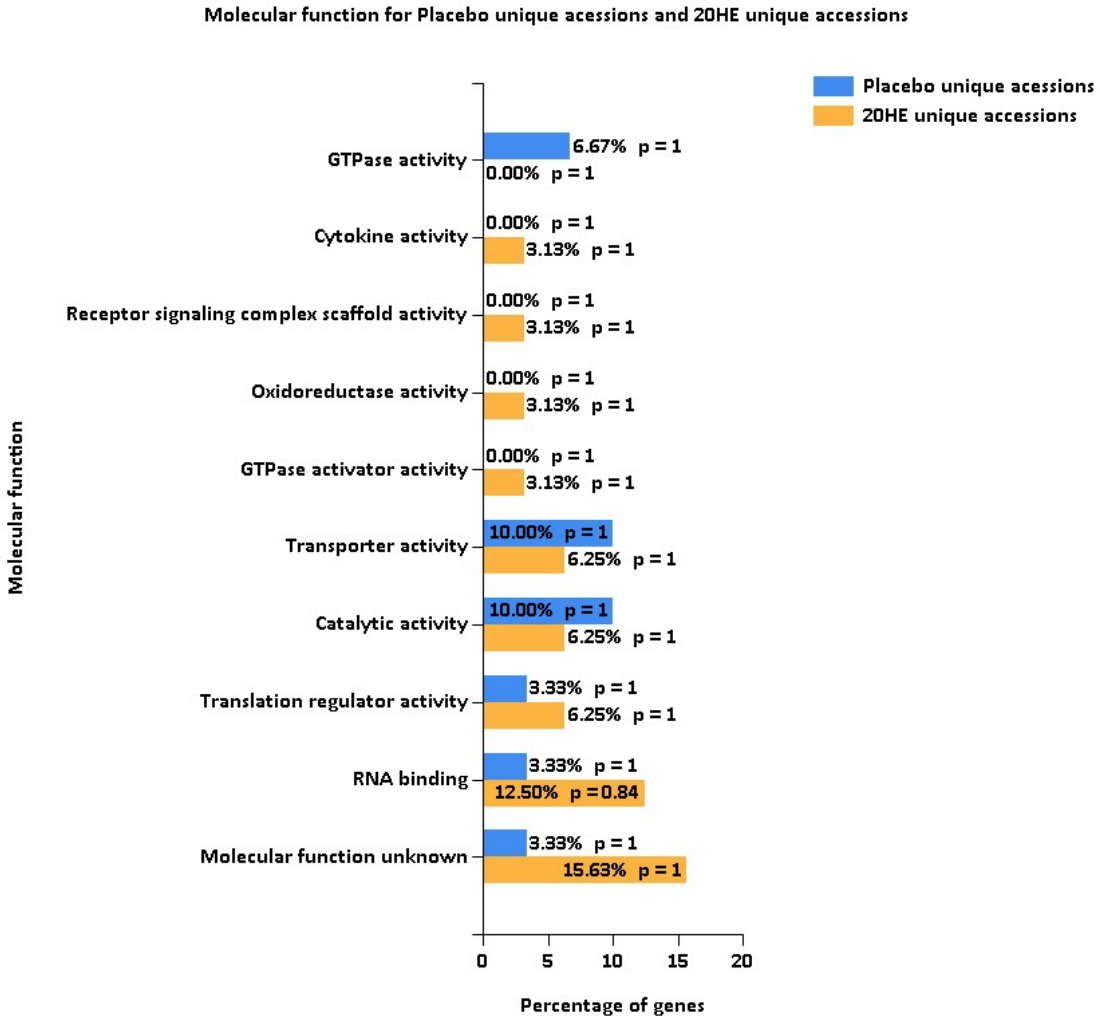
(Table-26) Fifteen out of 36 genes that expressed different proteins in response to 20HE. Proteome data showed proteins only detected in either treatment groups and after converting the protein accession numbers to their respective gene names, 36 proteins from both groups were expressed from the same gene.

Proteins accession number lists were used comparing unique proteins detected in the placebo group to proteins uniquely detected in the 20HE group. Functional enrichment analysis on biological processes (Figure-58). Cell growth and or maintenance pathway was represented by little over 15% in the 20HE treated cohort in contrast to the placebo group which was 3.33%. Protein metabolism and signal transduction pathways were also more enriched after 20HE treatment compared to the placebo group (Figure-58).

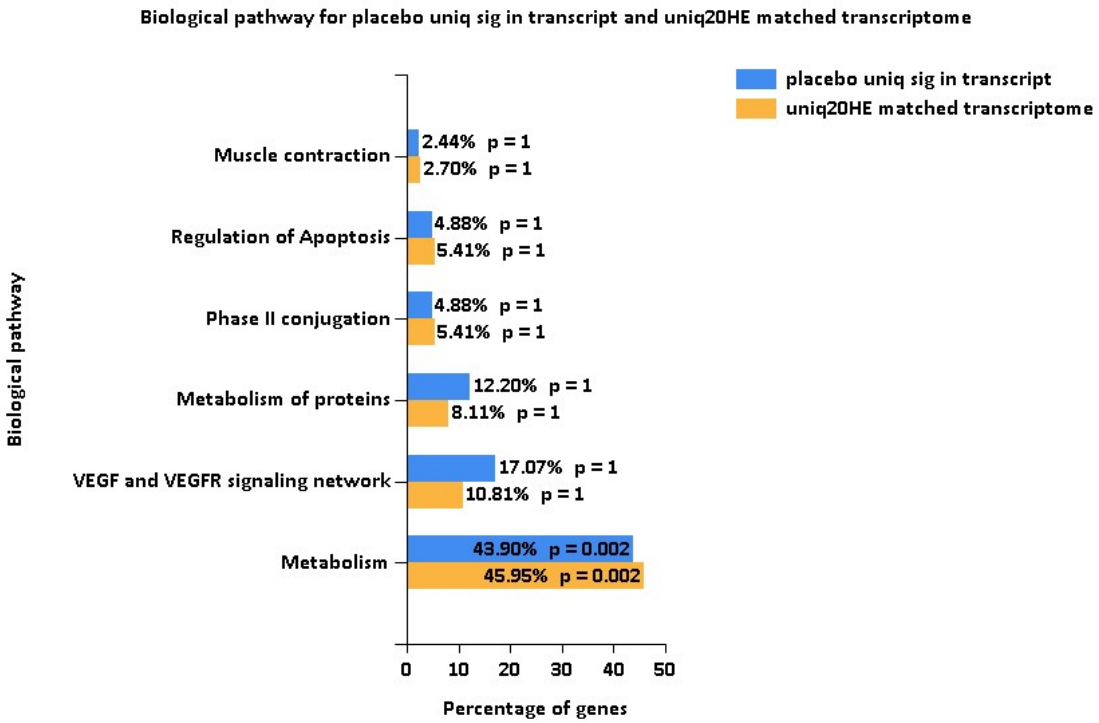


(Figure-58) Protein lists uniquely detected in either 20HE or the placebo group were analysed using FunRich software v. 3.1.3 and few of the pathways enriched in each cohort is presented. In the 20HE group 25% of the genes only detected in this group were related to signal transduction and cell communication while almost 19% of these proteins were associated with protein metabolism.

Another analysis was conducted comparing molecular function (Figure-59). Increased representation of catalytic and GTPase activity was observed in the placebo group compared to the 20HE group. While GTP activator activity was increased in the 20HE group. Proteins uniquely detected in either of the cohort (20HE or placebo treated) were also compared to the significantly differentially expressed genes from the transcriptome. Out of 59 proteins of the placebo group 58 were significantly differentially expressed in the transcriptome data while all 56 proteins were found also significant in the transcriptome data. After matching the proteins lists to the significantly expressed genes another functional enrichment analysis was conducted (Figure-60).



(Figure-59) Functional enrichment analysis of proteins uniquely expressed in either the 20HE or placebo group. Increased molecular function and oxidoreductase activities were observed in response to 20HE.

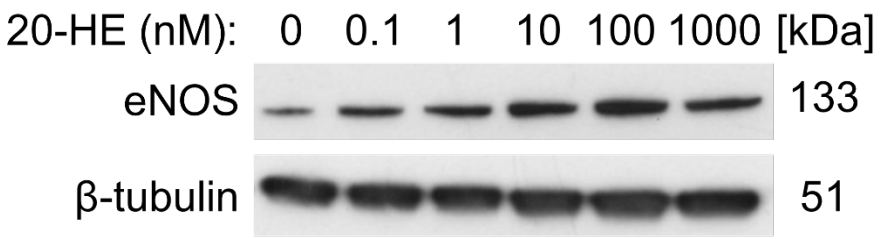


(Figure-60) Proteins uniquely expressed in either the 20HE or placebo treated cohorts were compared to their respective genes and those that were significant in the transcriptome data were included in this analysis. Metabolism pathway is significantly over represented in this data.

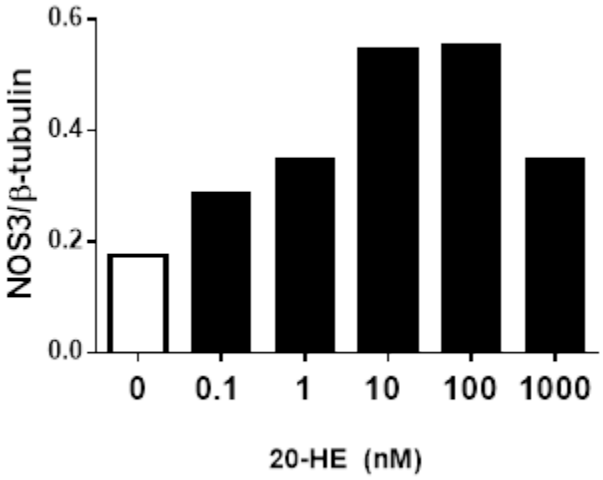
4.3 Validation of NOS3 expression

Throughout the course of this study Nitric oxide synthase 3 (NOS3) through the different techniques of investigation was consistently found significantly overexpressed. This was observed in the human microarray analysis, in the transcriptome data and in the functional enrichment analysis conducted represented by the VEGFR-signalling pathway. To further validate this finding, 20HE-induced increase in NOS3 protein expression in Human Coronary Artery Endothelial Cells (HCAECs) was investigated. HCAECs were treated with varying concentrations of 20HE which resulted in dose-dependent increases in NOS3 protein expression in cells treated with 20HE compared with the untreated control. An increase of 39% was recorded in cells treated with 1nM and highest increase of 68 % was recorded in cells treated with 100nM of 20HE compared with untreated control (Figure-61). qPCR data showed significant increases in NOS3 expression in response to increasing concentrations of 20HE (Figure-62).

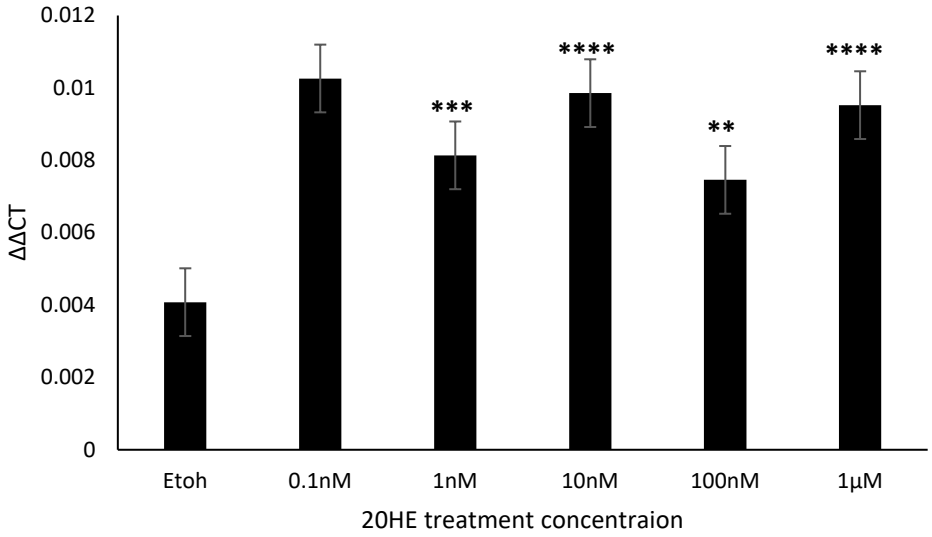
a)



b)



(Figure-61) Dose dependent effect of 20HE on NOS3 expression in HCAECs. At the level of protein noticeable increase in expression is observed in the gel image (a) and quantification of the Western blot normalised against β-tubulin (b).



(Figure-62) qPCR of NOS3 expression in HCAECs after treatment with different concentrations of 20HE. Significant increase in expression was observed in response to 1, 10, 100 nM and 1µM concentrations of 20HE with *p*-values of 0.004, 0.001, 0.02 and ≤ 0.001 respectively.

4.4 Discussion of results

Human physiological effects

The number of supplements containing ecdysteroids are increasing and often advertised either as natural steroids or in the form of their natural sources such as spinach, Suma root or quinoa. This prompts for further investigation of its effect in humans especially since its ergogenic effect appeals to a great portion of society. Physiological effect of 20HE in humans *in-vivo* as a supplement (Desire-X) that is readily available from online retailers was reported in this chapter.

After the intervention with 20HE supplement an increase in body weight was observed that is attributed to increased muscle mass since the fat mass was reduced by ≈1 fold. Measures of blood pressure showed significant decrease in DBP which affected the calculated MAP to be reduced but did not reach significance. Blood chemistries were also recorded, and Creatinine was found to be significantly increased after the 20HE intervention *p*-value= 0.03.

Indices of aerobic fitness did not show any significant improvement (VO_{2max}, vVO_2 and MET). Significant decrease in MVV and RQ were recorded after the intervention with 20HE. MVV (Maximum voluntary ventilation) is considered as an indication ventilatory function and RQ (respiratory quotient) value indicates the amount of energy used for metabolic processes and values of less than 1 indicate the use of protein and carbohydrates as a source of energy. MVV and RQ measurements were significantly reduced (p <0.01) after the intervention with 20HE while in the placebo supplement group MVV was significantly reduced (p <0.01) and RQ was also reduced without statistical significance.

Human *in-vitro* study on mitochondrial membrane potential (MMP)

Mitochondrial membrane potential was investigated *in-vitro* in response to varying concentrations of 20HE. Significant increase in MMP was observed after overnight treatment of the cells with 20HE at concentration of 5 & 10µM. No difference was observed after treatment with 1µM of 20HE. These experiments were conducted on preadipocytes and myoblasts and technical difficulties caused the inability to differentiate these cells to investigate MMP on mature adipocytes and skeletal muscle cells.

Molecular mechanisms

The first omics approach was the proteome analysis which quantified the abundant proteins within the liver tissue of 20HE treated and placebo treated groups. Proteomics analysis method used looked at the total proteins without depletion of the most abundant proteins. It was reported that 99% of proteins extracted from blood sera comprised of only 22 proteins (95). Nonetheless, using quantification method allowed the comparison between the two groups where, out of 1664 proteins identified and quantified, 68 were uniquely detected in the placebo group and 70 were unique to the 20HE treated group. The second omics approach was the transcriptome analysis using RNA extracted from liver tissue of 20HE and placebo treated groups. Analysing the data on TAC 4.0 software presented 37 differentially expressed genes with significance (p-value≤ 0.05) which included significant decrease in the expression of a negative regulator of myogenesis (HES1).

Omics approaches yield an overwhelming amount of data which can be challenging to analyse. Functional enrichment analysis allows the visualisation of these data in the context of their biological functions which can provide an indication of possible pathways of interest with ease. Functional enrichment analysis was conducted on both protein and gene lists extracted from the proteome and the transcriptome. Metabolism pathways were enriched in the proteome while few pathways were identified and explored including the VEGFA-VEGFR signalling pathway from the transcriptome data. Protein lists were then converted to their gene ID and the resulting gene lists from the proteome were analysed against the transcriptome data. This combined approach showed that the proteins that were uniquely detected in either group (20HE/placebo treated) were expressed from the similar genes.

ranscriptome analysis can only provide an indication of significant upregulation of a gene and the actual protein that exerts the physiological function remains unknown until a combined method as conducted in this study is done. Transcripts undergo the process of alternative splicing which allows a single gene to produce different proteins or different isoforms of a protein. It's been reported that the function of isoforms of the same protein translated from the same gene can cause conflicting effects in the context of cellular processes (96). According to another paper the different isoforms of a protein can exhibit striking difference in their interactome networks that it may appear to be originated from different genes (97). This shows the complexity of data analysis from the omics approach and how a combination of two or more platforms can provide more accurate indication of physiological functions. In addition, analysis complexity is expectedly great where protein IDs can be analysed against each other (treatment vs placebo) or against gene lists (treatment vs placebo vs transcriptome, treatment vs transcriptome,

placebo vs transcriptome, unique 20HE proteins vs transcriptome and unique proteins vs transcriptome) and finally protein IDs converted to

their gene names and compared against all the aforementioned analysis possibilities. In numbers, the following numbers of gene were matched to the transcriptome; 714 genes converted from total proteome, 56 genes from proteins unique to 20HE treated group and 61 genes from proteins unique to placebo treated group. Finally, after converting uniquely expressed genes in either group, 36 of these proteins originated from the same genes.

Validation nos3

Another finding that was further investigated, analysed and validated was the NOS3 overexpression in response to 20HE. Multiple techniques used during this study indicated the effect of 20HE on vasculature due to the significant overexpression of NOS3 in response to the pure form of the drug and as a supplement where 20HE also significantly affected the diastolic blood pressure. This was validated and confirmed *in-vitro* on human coronary artery endothelial cells.

CHAPTER 5

DISCUSSION, CONCLUSIONS AND FUTURE WORK

The global market for dietary supplements is estimated at be over $ 100 billion, which acts as an enormous incentive for manufacturers to try new and improved formulations continuously to ensure a competitive edge. Clinical trials on new preparations and personalisation of nutrition are just few of the recent trends within this industry (76). Though dietary supplements are intended to complement balanced diets, many consumers take a supplement without considering whether they actually need it, mainly because these supplements do not require prescriptions. Also, many of these products are actively advertised to the general population. Ergogenic effects of certain supplements appeal to a general cohort of consumers, while performance enhancing substances appeal especially to athletes, both recreational and elite. In the professional competitive sports context, dietary supplementation has increased the threats of inadvertent doping and also the risks to health. Many reports dispute the need or even the effectiveness of dietary supplements in performance enhancement, and, actually propose that it can, far more safely and effectively, be replaced with a suitably balanced diet, designed specifically for the nutritional demands before, during and after exercise, which could provide the required nutrition of the body when needed (98). It is understandable to recognise the pressure on elite athletes to enhance their performance, even marginally, which explains the “culture of additivity” (98). This has led to increased supplement intake among athletes, where good supplementation is linked to enhanced performance. An increasingly affluent and image conscious young population are also very susceptible to the lure of the quick fixes offered by these supplements.

5.1 Discussion of results. As discussed in chapter 1, the labels on supplements do not necessarily reflect their actual contents, and this was supported by the results of this study, where around 69% of the obtained supplements that claimed to contain 20HE had unquantifiable or undetectable amounts of the ecdysteroid . These findings have been confirmed by another recent study on supplement-derived 20HE (99), which showed that the concentration of 20HE in various supplements can vary enormously. Mostly, the concentrations specified on the labels were much higher than those obtained by analysis (99). This may be because reference materials and standards are only available for a few ecdysteroids from reputable suppliers, such as the LGC Group and Sigma Chemical Company, for 20HE, Ponisterone A and Muristerone. Without these calibrants and quality control compounds it is not possible to set up specific assays or even test for cross-reactivity, especially for the more potent anabolic species, such as Turkerterone (55). A more accurate reporting of concentrations and the specific ecdysteroid on the label is imperative in sports supplements, since inadvertent doping cases is still reported as substance abuse.

The investigation of these supplements after in vivo ingestion is also crucial and the use of the uPA +/+ SCID mouse model has proven informative for the hepatic metabolism of Methandrostenolone and 20HE, and, therefore can

be utilised for pharmacokinetic studies and analysis of the parent compound and its metabolites as biomarkers of use. However, it has to be recognised that this murine model is not without its limitations, such as the inability to investigate whole body physiology in these immunocompromised mice and the irrelevance of such an investigation if carried out, as the liver is the only humanised part of the animal. In addition, using this model provides only a limited time of exposure to the drug, thus does not lend itself to the investigation of chronic effects, as, for ethical reasons these animals cannot be maintained for more than 5 days. It is well documented that athletes consume supplements for long durations and this model cannot reflect that. On the other hand, excretion data of 20HE analysed from the animal model was confirmed and extended by the human excretion study, where 20HE remained detectable, at higher doses, for up to 48 hours after ingestion, which is an important finding in the context of doping control, since 20HE was added to the WADA monitoring list in October 2019. As it is likely that much higher doses are used by athletes because of their anabolic effects (66), the current data suggest that the detection window may be extended beyond 48 hours.

Ecdysteroids appeal to its consumers due to their reported anabolic effects, that include the reduction of fat mass and increased muscle mass, which was also observed in this study but did not reach statistical significant, perhaps due to the lower doses used here. A major finding in the physiological studies in the human volunteers was the reduction in diastolic blood pressure. How and if this may affect blood flow needs further investigation.

Results from the uPA +/+ mice (liver) and human studies (peripheral blood cells) showed a significant upregulation of the NOS3 gene, which encodes for endothelial Nitric Oxide Synthase (eNOS) enzyme (chapters 3 and 4). This was later confirmed in-vitro using human coronary artery endothelial cells (HCAECs, chapter 4). Nitric oxide is a known potent vasodilator aiding in providing adequate blood supply, which is imperative during physical activity (100). While this vasodilatory effect may attract professional athletes to ensure adequate blood supply during competition it is important to note that literature showed that these effects of 20HE are not targeted, meaning that neither the vasodilatory effect nor the anabolic effects are specific to skeletal muscle (54). Therefore, increased blood flow at rest or in other organs, may not allow a directed response during exercise aimed specifically at increased skeletal muscle blood flow, which may actually hinder performance. In addition, while 20HE may increase blood flow due to this vasodilatory effect, it has actually significantly reduced red blood cell (RBC) count (p= 0.01), which is another crucial parameter for oxygen delivery during performance of sport, which is why many athletes choose to undergo homologues or autologous blood transfusion, in order to increase their red cell count during competition (101).

Its effects on mitochondrial membrane potential was significant indicating higher production of ATP. Although these data were from preadipocytes and myoblasts and further investigation on mature cells is required,

nonetheless aerobic fitness was also improved *in-vivo* in humans after 20HE intervention. This finding is very relevant in competitive sports, where any marginal improvement in performance is sought. One oversight while investigating aerobic fitness was the inability to calculate the aerobic threshold or ventilation threshold (AT) which is considered as a measure of fitness. In brief, a basic response to exercise is ventilation which increases linearly as the intensity of exercise and oxygen consumption are increased (aerobic metabolism) up to a threshold after which anaerobic metabolism is induced. AT the highest intensity an individual can maintain for a relatively long period of time without fatigue or build-up of lactic acid that may happen when AT is exceeded. It is measured as V_{CO2}/V_{O2} and shall be included in future studies measuring aerobic fitness (25). Other improvements included blood glucose and increased healthy cholesterol and decreased LDL cholesterol.

Another interesting finding from the transcriptome studies was the downregulation of CYP17A1 in the 20HE treated liver. The CYP17A1 gene codes for making a protein belonging to the cytochrome P450 enzyme family. Similar to other cytochrome P450 enzymes, CYP17A1 regulates the synthesis of steroid hormones, such as testosterone and oestrogen, as well as the mineralocorticoids and glucocorticoids. The CYP17A1 enzyme catalyzes two key reactions, one associated with 17 alpha(α)-hydroxylase activity, converting pregnenalone to 17-hydroxypregnenolone and progesterone to 17-hydroxyprogesterone (102), and the other is its 17,20-lyase activity, which converts 17-hydroxypregnenolone to dehydroepiandrosterone (102; DHEA). This reaction is a rate limiting step in the production of sex hormones. How, and if, this might be a role of 20HE, leading to alterations in levels of hormones such as testosterone is as yet unclear. It has been shown that CYP17A1 is highly expressed in over 50% of human prostate carcinomas, perhaps pointing to a role for it in intracellular androgen synthesis by cancer cells (103). Abiraterone, an FDA approved CYP17A1 inhibitor, has been reported to block nuclear accumulation of AR (104), but, whether this can be mimicked by 20HE has not been investigated, either in vitro or in vivo.

5.2 Limitations of the studies.

It is well understood that each study comes with its own set of limitations and in this study the limitations with regards to the use of the uPA +/+ SCID mouse model to investigate human hepatic metabolites were briefly discussed where the animals can only remain viable for up to 5 days, constricting any investigation to immediate effects only, without the ability to study the chronic effects on the liver metabolism and liver tissue. Systemic markers, effects on other organs, such as the adipose tissue and skeletal muscle, as well as whole body physiological effects of 20HE could not be investigated using this model, as organs other than the liver are not

humanised and therefore information acquired from such investigations would be reliable, due to the nature of the animals.

The human *in-vivo* investigations used 20HE supplementation, which was only one of the constituents of the supplement, rather than the pure form of the drug. This is a limitation as the other components may alter the way 20HE is absorbed or works. Therefore, while using supplement-derived 20HE may have allowed the ethical investigation of the ecdysteroid in humans in-vivo, that some of the effects seen may have been caused by other constituents of the supplement, at least in part or in conjunction with 20HE, cannot be ruled out. This is especially so since the placebo supplement used was chosen only for its lack of detectable quantity of any ecdysteroid, and not a true placebo where its constituents are identical to the test supplement, but only lacking the drug of interest.

The number of volunteers in the study were low. This was because of a high fall out rate and low compliance. As there was a need to recruit recreational athletes with a good level of aerobic fitness, so as to be of at least some relevance to the elite athletes, this posed a limiting factor to the numbers of volunteers identified. Therefore, this can only be viewed as a pilot study that needs to be extended and confirmed in larger numbers. In addition, only clinic blood pressure was recorded, and as the reduction in diastolic blood pressure was an interesting finding, this needs to be repeated with more precise techniques.

In-vitro investigation of mitochondrial membrane potential was conducted using a single wavelength fluorescence measurement where the reduction in the of the emission intensity may have resulted from dye leakage or reduction of cell number due to toxicity and not necessarily an alteration of membrane potential per se.

5.3 Future research.

Many recreational and elite athletes may be using ecdysteroids due to their anabolic effects, making grounds for future investigation on the prevalence of ecdysteroids consumption among athletes. It would be possible to investigate this retrospectively using the methods developed in this study. The WADA accredited anti-doping laboratories collect 2 urine samples (A & B samples) on and off competition from competing athletes. The A sample is analysed to detect any known doping agent using various established methods. Once the results are reported as being negative the B samples are stored for only 3 months before being discarded. A future study could utilise these B samples, that were reported negative for current WADA prohibited substance, for this retrospective study to calculate prevalence of use. This is possible because all competing athletes have provided, signed informed consent for the future use of their samples for anti-doping research.

The induction of NOS3 by 20HE needs to be investigated further in peripheral blood cells and skeletal muscle biopsies from trained athletes at rest and after training both acutely and over a period of time, along with more detailed measures of blood pressure and flow.

The inhibition of the expression of CYP17A1 by 20HE and its putative effect on systemic and tissue levels of steroids, such as oestrogen, progesterone, DHEA and testosterone needs to be investigated. There is some indirect evidence that ecdysteroids may be associated with steroid hormone disruption (63). This offers scope for 20HE beyond sport, especially in the treatment of cancers, such as prostate and breast.

Key experiments to follow-on the findings include:

- Further investigation on the physiological effects of supplement derived 20HE in humans *in-vivo* with higher numbers and using true placebo supplement spiked with 20HE
- Confirmatory and addition investigation of 20HE effects on mitochondrial respiration using Seahorse XF analyzer to test mitochondrial function in addition to oxygen consumption method.
- In-depth analysis of the differential effects caused by 20HE from the proteome and transcriptome data bith separately and as a combined data analysis.

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Appendices

Appendix-1

World Anti-Doping Agency (WADA) publishes a list of substances as part of their monitoring program which are not yet prohibited. It includes substances that are observed for possible misuse in sports.

THE 2020 MONITORING PROGRAM*

The following substances are placed on the 2020 Monitoring Program:

1. **Anabolic agents:**	*In-* and *Out-of-Competition*: ecdysterone
2. **Beta-2-agonists:**	*In-* and *Out-of-Competition*: any combination of beta-2-agonists *In-* and *Out-of-Competition*
3. **2-ethylsulfanyl-1H-benzimidazole (bemitil):**	
4. **Stimulants:**	*In-Competition* only: bupropion, caffeine, nicotine, phenylephrine, phenylpropanolamine, pipradrol and synephrine
5. **Narcotics:**	*In-Competition* only: codeine, hydrocodone and tramadol *In-Competition* (by routes of administration other than oral,
6. **Glucocorticoids:**	intravenous, intramuscular or rectal) and *Out-of-Competition* (all routes of administration)

*The World Anti-Doping Code (Article 4.5) states: "WADA, in consultation with Signatories and governments, shall establish a monitoring program regarding substances which are not on the Prohibited List, but which WADA wishes to monitor in order to detect patterns of misuse in sport."

Appendix-2

Qiagen RT2 profiler arrays were used to investigate phase I drug metabolism enzymes. They included 84 key genes involved in the process in addition to housekeeping genes for normalisation.

a)

AADAC	ADH1A	ADH1B	ADH1C	ADH4	ADH5	ADH6	ADH7	ALDH1A1	ALDH1A2	ALDH1A3	ALDH1B1
ALDH2	ALDH3A1	ALDH3A2	ALDH3B1	ALDH3B2	ALDH4A1	ALDH5A1	ALDH6A1	ALDH7A1	ALDH8A1	ALDH9A1	CEL
CYP11A1	CYP11B1	CYP11B2	CYP17A1	CYP19A1	CYP1A1	CYP1A2	CYP1B1	CYP21A2	CYP24A1	CYP26A1	CYP26B1
CYP26C1	CYP27A1	CYP27B1	CYP2A13	CYP2B6	CYP2C18	CYP2C19	CYP2C8	CYP2C9	CYP2D6	CYP2E1	CYP2F1
CYP2R1	CYP2S1	CYP2W1	CYP3A4	CYP3A43	CYP3A5	CYP3A7	CYP4A11	CYP4A22	CYP4B1	CYP4F11	CYP4F12
CYP4F2	CYP4F3	CYP4F8	CYP7A1	CYP7B1	CYP8B1	DHRS2	DPYD	ESD	FMO1	FMO2	FMO3
FMO4	FMO5	GZMA	GZMB	HSD17B10	MAOA	MAOB	PTGS1	PTGS2	UCHL1	UCHL3	XDH
ACTB	B2M	GAPDH	HPRT1	RPLP0	HGDC	RTC	RTC	RTC	PPC	PPC	PPC

b)

Aadac	Adh1	Adh4	Adh5	Adh7	Aldh1a1	Aldh1a2	Aldh1a3	Aldh1a7	Aldh1b1	Aldh2	Aldh3a1
Aldh3a2	Aldh3b1	Aldh3b2	Aldh4a1	Aldh5a1	Aldh6a1	Aldh7a1	Aldh8a1	Aldh9a1	Cel	Cyp11a1	Cyp11b1
Cyp11b2	Cyp17a1	Cyp19a1	Cyp1a1	Cyp1a2	Cyp1b1	Cyp21a1	Cyp24a1	Cyp26a1	Cyp26b1	Cyp26c1	Cyp27a1
Cyp27b1	Cyp2a4	Cyp2a5	Cyp2c29	Cyp2c38	Cyp2c39	Cyp2c54	Cyp2c55	Cyp2c65	Cyp2c66	Cyp2d22	Cyp2e1
Cyp2f2	Cyp2r1	Cyp2s1	Cyp3a11	Cyp3a13	Cyp3a16	Cyp3a25	Cyp3a44	Cyp3a57	Cyp4a10	Cyp4a12a	Cyp4b1
Cyp4f14	Cyp4f15	Cyp4f18	Cyp7a1	Cyp7b1	Cyp8b1	Dhrs2	Dpyd	Esd	Fmo1	Fmo2	Fmo3
Fmo4	Fmo5	Gzma	Gzmb	Hsd17b10	Maoa	Maob	Ptgs1	Ptgs2	Uchl1	Uchl3	Xdh
Actb	B2m	Gapdh	Gusb	Hsp90ab1	MGDC	RTC	RTC	RTC	PPC	PPC	PPC

Phase 1 drug metabolising panel. a) Human PCR Array. RT² Profiler™ PCR Array Human Drug Metabolism: Phase I Enzymes (PAHS-068Z) – Qiagen and b) **Mouse PCR Array.** RT² Profiler™ PCR Array Mouse Drug Metabolism: Phase I Enzymes (PAMM-068Z) – Qiagen

Appendix-3

Human *in-vivo* studies investigating the human physiological effects of 20HE were conducted. Volunteer's criteria for inclusion or exclusion from the study are summarised in the table below.

Criteria for	Description
Inclusion	Male, Adult (aged ≥ 21 years), Recreational athletes, BMI ≤ 30 Kg/ m2, of Arab ethnicity
Exclusion	Diabetes, Hypertension, taking dietary supplements in the 3 months preceding the study, Current treatment with warfarin, Connective tissue disease or other inflammatory conditions likely to affect cytokine levels, Malignancy or other terminal illness, Immune compromised subjects, Autoimmune disease, Chronic infection, Substance abuse or other causes of poor compliance, VO2 max ≤ 30 ml/kg/min

Appendix-4

Consent form signed by the recruited volunteers for the human *in-vivo* study.

Participant's Statement

Documentation of Permission

Principal investigator

As a representative of this study, I have explained the purpose, the procedures, the possible benefits and risks that are involved in this research study. Any questions that have been raised have been answered to the individual's satisfaction.

Signature of Person Obtaining Consent	Date of Signature

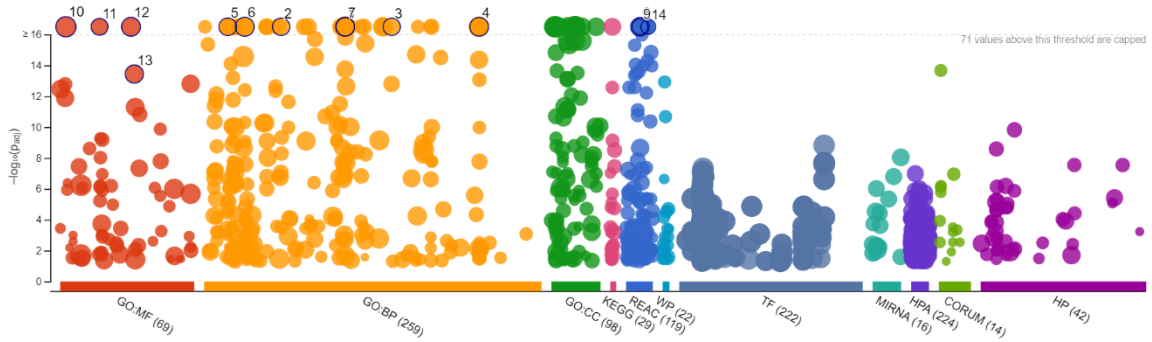
Research Participant / Authorized Persons

I, the undersigned have been informed about this study's purpose, procedures, possible benefits and risks, and I have received a copy of this consent. I have been given the opportunity to ask questions before I sign, and I have been told that I can ask other questions at any time. I voluntarily agree to be in this study. I am free to stop being in the study at any time without need to justify my decision and if I stop being in the study I understand it will not in any way affect the benefits I normally have. I agree to cooperate with (name of principal investigator) and the research staff and to tell them immediately if I experience any unexpected or unusual symptoms.

Participant's Signature	Date of Signature
Signature of Witness	Date of Signature
Signature of Legally Authorized Representative (When Appropriate)	Date of Signature
Relationship to Participant (When Appropriate)	Date of Signature

Appendix-5

Functional enrichment analysis conducted on the gene list exported from the Transcriptome and proteome data. genes that were significantly altered in expression according to the transcriptome data and matched with their respective proteins according to the proteome data. X-axis shows the databases the gene list was ran against and the y-axis shows the adjusted *p*-value. Detailed interactions are presented in the table below.



ID	Source	Term ID	Term Name	p_{adj} (query_1)
1	GO:BP	GO:0044281	small molecule metabolic process	1.616×10^{-49}
2	GO:BP	GO:0019752	carboxylic acid metabolic process	8.776×10^{-47}
3	GO:BP	GO:0055114	oxidation-reduction process	5.391×10^{-46}
4	GO:BP	GO:1901575	organic substance catabolic process	1.116×10^{-44}
5	GO:BP	GO:0006082	organic acid metabolic process	7.163×10^{-44}
6	GO:BP	GO:0009056	catabolic process	6.813×10^{-42}
7	GO:BP	GO:0044248	cellular catabolic process	5.971×10^{-40}
8	REAC	REAC:R-HSA-14...	Metabolism	2.286×10^{-54}
9	REAC	REAC:R-HSA-71...	Metabolism of amino acids and derivatives	1.409×10^{-33}
10	GO:MF	GO:0003824	catalytic activity	3.271×10^{-30}
11	GO:MF	GO:0016491	oxidoreductase activity	5.924×10^{-29}
12	GO:MF	GO:0036094	small molecule binding	2.761×10^{-17}
13	GO:MF	GO:0042802	identical protein binding	3.646×10^{-14}
14	REAC	REAC:R-HSA-37...	Signaling by ROBO receptors	4.047×10^{-19}

version	e100_eg47_p14_7733820
date	8/12/2020, 10:49:09 AM
organism	hsapiens

g:Profiler